REMARKS

Claims 1, 3, 5-10, and 13-18 are canceled herein. Claims 21-37 are added. Support for the claims is found throughout the specification, and therefore it is believed that no new matter is added.

Formal matters

Applicants gratefully acknowledge the withdrawal of the objection to the specification and various rejections.

New claims

The instant claimed methods provide a powerful and efficient method to isolate the nucleotide sequences of antibodies or antigen binding fragments thereof, that are specific for a discreet peptide sequence using libraries of antibodies or antigen binding fragments thereof in combination with solid phase-dependent peptide synthesis. Using a set of overlapping and nonoverlapping oligopeptides from a target antigen, the claimed methods avoid the arduous selection process required for hybridoma creation and characterization, and eliminate the selection limits associated with immunodominant epitopes while expanding the range of novel antibodies available for selection in a rapid and specific manner.

The new claims find support throughout out the specification and specifically at, *inter alia*, page 6, lines 33-page 9, line 25; page 10, lines 5-21; page 11, lines 25-29; page 12, line 1-page 14, line 15, and page 14, line 19-page 17, line 3. The new claims were added to clarify the scope of the claimed methods. Further, Applicants retain the right to pursue claims to any subject matter outside the scope of the instant claims in future related applications.

Rejection Under 35 U.S.C. § 112, first paragraph - written description

Heterologous oligopeptides

Claims 1, 3, 5-10, and 13-18 were rejected under 35 U.S.C. § 112, first paragraph as lacking sufficient written description in the specification to reasonably convey to a person of skill in the art possession of the set of heterologous peptides used in the claimed methods at the time of

filing. *See* Final Office Action dated July 14, 2004 at 2. According to the Examiner, the specification provides support for a nonoverlapping set of multimers rather than a heterologous set of oligopeptides. *See id* at 3. The Examiner specifically asserts that the term "heterologous" encompasses the use of heterologous oligopeptides that can be derived from different target protein of different species. *See id.* Applicants traverse this rejection.

While Applicants believe there is ample support for the term "heterologous", the instant rejection is moot in view of the new claims submitted herewith.

Synthesizing Step

Claims 1, 3, 5-10, and 13-18 were rejected under 35 U.S.C. § 112, first paragraph as lacking sufficient written description in the specification to reasonably convey to a person of skill in the art possession of the claimed methods that includes the steps of synthesizing a set of heterologous oligopeptides derived from a target protein at the time of filing. According to the Examiner, the specification relates to a specific set of 25 nonoverlapping peptides that span the CD64 extracellular domain in the disclosed example. *See* Final Office Action dated July 14, 2004 at 3. The Examiner asserts that the single example is insufficient because the specification lacks any description of structure or sequence, citing the Federal Circuit's standard for adequate description of a chemical genus. *See id.* at 4. Applicants traverse this rejection.

Applicants respectfully submit that the specification's disclosure regarding the step of synthesizing heterologous, *i.e.*, overlapping and nonoverlapping, oligopeptides is sufficiently described to fulfill the written description requirement of 35 U.S.C. § 112, first paragraph. "An adequate written description of the invention may be shown by **any description** of sufficient, relevant, identifying characteristics so long as a person skilled in the art would recognize that the inventor had possession of the claimed invention." MPEP § 2163(II)(3)(a) (emphasis added). While the disclosed embodiment describes the use of nonoverlapping peptides, Applicants readily demonstrate possession of the claimed invention in the description of the synthesizing step in the specification. Specifically, the specification discloses the synthesis of peptides on polystyrene rods or other solid phases such as membranes or beads. *See* the specification at page 7, lines 20-23. The specification further discloses the use of pepscan technology as an exemplary method for the synthesis of oligopeptides on solid phase. *See id.* at page 7, lines 23-24. The specification also

describes pepscan technology. *See id.* at page 8, lines 1-9. The specification further delineates the identity of the synthesized peptides as "individual peptides, the amino acid of which is determined by the amino acid sequence of the target protein" as well as providing preferred size ranges for the oligopeptides. *See id.* at page 13, lines 14-17 and page 7, lines 33-35, respectively. At the time of filing, pepscan technology had been used in a wide range of applications. *See, e.g.*, Exhibit A at page 36. Thus, a person of ordinary skill in the art would readily recognize the type of technology encompassed by synthesizing step of the claimed methods in view of the disclosure of pepscan technology as a preferred embodiment for the synthesizing step given the well known nature of the synthesizing technology. *See* MPEP § 2163 (II)(3)(a) ("What is conventional or well known to one of ordinary skill in the art need not be disclosed in detail. [] If a skilled artisan would have understood the inventor to be in possession of the claimed invention at the time of filing, *even if every nuance of the claims is not explicitly described in the specification*, then the adequate description requirement is met.") (citations omitted) (emphasis added).

Finally, the claimed methods are not a chemical invention, and therefore the written description provided by the specification is sufficient. The invention of the claimed methods lies not in the synthesis of the oligopeptides on a solid phase, but in a particular use of the synthesized peptides. The invention does not lie in the identification of novel proteins, peptide sequences, or peptide conformation and thus is not a chemical invention *per se*. Rather the invention lies in the ability to synthesize an oligopeptide library that represents all or a portion of a target protein in small fragments so that the nucleotide sequences of antibodies specifically reacting to a specific peptide can be readily identified and rapidly expanded. The guidance provided in the specification combined with the knowledge in the art is sufficient to convey to the skilled artisan that Applicants had possession of the invention at the time of filing, therefore the written description requirement of 35 U.S.C. § 112, first paragraph is met.

For at least these reasons, the written description rejections addressed above under may be withdrawn.

Rejection Under 35 U.S.C. § 112, second paragraph

Single chain antibody fragment and scFv

Claims 1, 3, 5-10, and 13-18 were rejected under 35 U.S.C. § 112, second paragraph as failing to particularly point out and distinctly claim the subject matter of the invention. The Examiner alleges that a single chain antibody fragment and a scFv are the same. *See* Office Action dated December 1, 2003 at 5 and Final Office Action dated July 14, 2004 at 5. The Examiner cites the instant specification at page 3, lines 29-31, asserting that the specification defines the single chain antibody fragment as a scFv. *See* Final Office Action dated July 14, 2004 at 5. The Examiner also asserts that no evidence of any single chain antibody fragment other than the scFv has been presented. *See id.* Applicants traverse this rejection.

Applicants respectfully submit that single chain antibody fragment and scFv are not synonymous, and therefore the use of the terms creates no indefiniteness in the claimed methods. As previously discussed in the Amendment dated April 21, 2004, the term "scFv" refers to a specific type of single chain antibody fragment. Neither the plain language nor its use in the art limits it to a single chain antibody fragment consisting of only of variable regions. First, the plain language of the term encompasses any single chain antibody fragment, including but not limited to scFv. Second, the term as typically used in the art encompasses more than scFv. *See, e.g.* Exhibit B at Abstract (stating that the article focuses on "single chain antibody fragment production (scFv and V_{HH})" in lower eukaryotes). Therefore, the terms are both clear and precise, and are not used in a way contrary to the art.

Furthermore, the meaning of the term "single chain antibody fragment" in the claims is not limited in the specification. The Examiner points to the following passage to assert that the specification defines single chain antibody fragment as an scFv:

> This has been achieved by making a fusion through a peptide linker between a heavy chain and a light chain subunit. This results in so-called single chain antibodies ...

See the specification at page 3, lines 29-31. There is nothing in this cited definition that limits these two subunits to a heavy chain variable region and a light chain variable region as in an scFv. Moreover, it is clear in the specification that single chain antibody fragments encompasses more than scFv. For example, original claim 9 reads:

> 9. A method according to anyone of the afore going claims whereby the specific binding peptide is a single chain antibody fragment, preferably an scFv.

See the specification at page 32. Therefore, the use of the cited terms in the claims are sufficiently definite.

Claim 3 duplicates claim 1

According to the Examiner, claim 3 is duplicate of claim 1 and therefore indefinite. Applicants traverse this rejection.

Claims 1 and 3 are canceled herewith, rendering this rejection moot.

Synthesizing step

Claims 1, 3, 5-10, and 13-18 were rejected under 35 U.S.C. § 112, second paragraph as failing to particularly point out and distinctly claim the subject matter of the invention. The Examiner asserts that the cited claims are indefinite as to the step of synthesizing a set of heterologous oligopeptides derived from the target protein. *See* Final Office Action dated July 14, 2004 at 6. According to the Examiner, the manner of synthesis is unclear because it does not refer to continuous or non-continuous, linear or secondary structure of a peptide sequence in one or more target proteins. *See id.* Applicants assert this rejection is in error.

The indefiniteness requirement of 35 U.S.C. § 112, second paragraph informs the public of the metes and bounds of the claimed invention such that infringement of the claims is clear. *See* MPEP § 2173. However, the breadth of a claim cannot be equated with indefiniteness. *In re Miller*, 441 F.2d 689, 169 U.S.P.Q.2d 597 (CCPA 1971). "If the scope of the subject matter is clear, and if applicants have not otherwise indicated that they intend the invention to be a scope different from that defined in the claims, then the claims comply with 35 U.S.C. § 112, second paragraph." MPEP § 2173.04.

Applicants respectfully submit that the step of synthesizing oligopeptides is sufficiently definite to inform the public of the boundaries of the claimed methods. As disclosed in the specification, the oligopeptides employed in the method may be linear or non-linear, continuous or non-continuous. *See, e.g.*, the specification at page 8, lines 7-9. The breadth of the type of oligopeptides useful with the claimed methods simply reflects the power of the claimed methods as

a platform to identify epitope-specific antibodies. It is well known that the epitopes recognized by antibodies can be linear or non-linear, continuous or non-continuous. *See, e.g.*, Exhibit C at page 1, second paragraph. Thus, antibody identification methods include a variety of different oligopeptides in a peptide library for screening, and reference to the library as an "oligopeptide library" introduces no improper ambiguity into the claim language. The synthesizing step as claimed is sufficiently definite to fulfill the patentability requirements.

For the reasons stated above, the above indefiniteness rejections under 35 U.S.C. § 112, second paragraph may be properly withdrawn.

Rejection Under the Judicially Created Doctrine of Obviousness-Type Double Patenting

Claims 1, 3, 5-10, and 13-18 were rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1, 5, and 6 of U.S. Patent No. 6,265,150 (the '150 patent) and claims 1, 5, and 6 of U.S. 2002/0132228 (the '228 application) in view of Middledorp et al (U.S. Patent No. 5,424,398). According to the Examiner, each of the '150 patent and the '228 application disclose the method as claimed except for the use of a set of oligopeptides as the target antigen. *See* Office Action dated December 1, 2003 at 8-9. The Examiner states that Middledorp teaches the synthesis of individual antigens or oligopeptides on solid support using the Geysen method. *See id.* The Examiner argues that Middledorp discloses the advantages of using sets of oligopeptides, the great importance of identifying immunodominant viral proteins and epitopes thereof, and the use of antigen fragments in these methods, therefore providing the motivation to employ sets of oligopeptides. *See id.* Applicants traverse this rejection.

Claims 1, 3, 5-10, and 13-18 are canceled herewith, rendering the above rejection moot.

Applicants further note that the '150 patent and the '228 application in view of Middledorp do not render new claims 21-34 obvious as the cited references fail to teach each and every element of the claimed invention. The Examiner asserts the following:

> "... contact and binding have been determined before the antibody can be identified. In any antigen-antibody reaction, binding needs to occur and identify before that specific antibody that is known to bind to antigen can be isolated."

See Final Office Action dated July 14, 2004 at 9-10. Applicants are unsure as to what is meant by this statement. It appears that the Examiner is asserting that the peptide specificity, *i.e.*, the actual peptide sequence within the antigen bound by the antibody, is readily determined, if not already known, using the combined teachings of the cited references. While the specific antibody is identified by its binding to the cell surface antigen on the cells disclosed in the '150 patent (and '228 application), these methods lack any teaching or suggestion of using overlapping or nonoverlapping oligopeptides to identify antibodies specific for those peptides. Rather the '150 patent and the '228 application are directed to the identification of antibodies that bind surface antigens in their native configurations on the cell. *See, e.g.*, the '150 patent at Abstract. Isolation of the nucleotide sequence for the antibody which binds a specific, known sequence is not required nor is such a method disclosed in these references.

Middledorp fails to cure the deficiency in the '150 patent and the '228 application because Middledorp is directed to the reverse concept of these patent documents, the identification of immunodominant viral proteins and epitopes. *See* Middledorp at, *e.g.*, col. 4:46-50. Thus, Middledorp focuses on determining what antigen and portion of an antigen is recognized by immune antiserum. *See, e.g., id.* at col. 4:60-63 (disclosing that peptides are particularly useful in diagnostic kits to determine the presence of anti-EBV antibodies in immune antiserum). In contrast, the '150 patent and '228 application relate to the use of whole cells to identify new antibodies that are not limited to those that are naturally occurring, *i.e.*, found in immune antiserum. Moreover, to modify Middledorp using the teachings of the '150 patent or '228 application would render the method of Middledorp unsuitable for its intended purpose. *See* MPEP § 2143.01 ("If [the] proposed modification would render the prior art invention being modified unsatisfactory for its intended purpose, then there is no suggestion or motivation to make the proposed modification.") (citations omitted). Contacting the peptides of Middledorp with the antibody phages of the '150 patent and '228 application fails to identify immunodominant peptides of EBV that are suitable for use in diagnostic kits to detect EBV infection. The identification of immunodominant epitopes relies on the use of immune antiserum, a step that is clearly eliminated in the '150 patent and '228 application. Thus, there is no suggestion or motivation to combine the cited references. In the absence of such a motivation, the cited references fail to render the claimed methods obvious.

The lack of motivation to combine the cited references is further evidenced in the Declaration of John de Kruif pursuant to 37 C.F.R. § 1.132 submitted herewith as Exhibit E. Dr. de Kruif describes the methods in the cited documents as nonoverlapping. *See* Exhibit E at ¶5. He further indicates that the modification of Middledorp to result in the claimed method results in the loss of Middledorp's primary function, *i.e.*, to identify immunodominant epitopes. According to Dr. de Kruif, this would not provide one of ordinary skill in the art with the motivation to combine these references.

For at least these reasons, the rejection under judicially created doctrine of non-obviousness may be withdrawn.

Rejections Under 35 U.S.C. § 103 (a)

de Kruif in view of Geysen and Granoff

Claims 1, 3, 5-10, and 13-18 were rejected under 35 U.S.C. § 103 (a) as being unpatentable over de Kruif et al., *J. Mol. Biol.*, 248:97-105 (1995) in view of Geysen et al., *J. Immunol. Methods* 102:259 (1987) and Granoff, U.S. Patent No. 6,030,619. According to the Examiner, de Kruif discloses a method of identifying scFv comprising constructing a library of phage display of scFv antibody fragments, Geysen discloses a set of heterologous oligopeptides, and Granoff teaches the conventionality of the method of antigen-antibody binding or reaction using different antibody and the different methods by which antigen that reacts with antibodies can be determined, *e.g.*, the Geysen method. Applicants traverse this rejection.

Applicants note that this rejection is very similar to the rejection made in earlier Office Actions where the Examiner cited de Kruif in view of Oirschot or Geysen and de Kruif in view of Middledorp, both rejections that were later withdrawn by the Examiner. *See, e.g.,* Office Action dated May 20, 2002 and Reply and Amendment dated September 4, 2002. In both rejections, the Examiner cited Middledorp and Geysen for their teaching regarding the use of a set of oligopeptides. In the instant action, Geysen is again cited for its disclosure of a set of oligopeptides while Granoff allegedly discloses the conventionality of using phage scFv with sets of oligopeptides in solid support. Applicants respectfully submit that the cited combination, as the others before it,

fails to provide the motivation to combine the teachings to result in the claimed methods and a reasonable expectation of success of such a combination for at least the follow reasons.

First, de Kruif lacks any disclosure teaching or suggesting the use of overlapping or nonoverlapping oligopeptides nor does de Kruif disclose the use of a set of heterologous antigen fragments as asserted by the Examiner. de Kruif discloses the use of whole antigens as well as an isolated domain from the von Willebrand factor, known as domain A2 or fragment A2. The A2 fragment or domain disclosed is one of a series of repeating domains within the complete protein and is 134 amino acids long. *See* Exhibit D at 1818. de Kruif discloses using the A2 domain as a single antigen to test several specific monoclonal phage antibodies after these antibodies were already panned against the whole von Willebrand factor (VWF). *See* de Kruif at page 102, first column, second paragraph. When phage antibodies were panned against the isolated A2 domain, it was used alone - as a whole domain on antigen-coated immunotubes. Thus, the disclosure of de Kruif contains nothing about using a set of overlapping or nonoverlapping peptides from a target protein.

Neither Geysen nor Granoff provide any motivation to combine their teachings with that of de Kruif to result in the claimed methods. First, Geysen is directed to the reverse concept of de Kruif. Geysen discloses a method of determining what subsequence of an antigen is "antigenically active" or "the antigenic determinant" using immune antiserum reactivity, or alternately stated, Geysen seeks to identify the immunodominant epitopes of an antigen. *See, e.g.*, Geysen at Abstract. In other words, Geysen focuses on determining what portion of an antigen is recognized by immune antiserum as opposed to de Kruif's use of whole antigens or antigen domains to generate additional antibodies. With Geysen's focus, there is no motivation to combine its disclosure with de Kruif because contacting the oligopeptides of an antigen with a plurality of antibody fragments of de Kruif would not help identify immunodominant epitopes. Moreover, such a modification would result in rendering the method of Geysen unsuitable for its intended purpose, *i.e.*, to identify epitopes recognized by immune antisera. *See* MPEP § 2143.01 ("If [the] proposed modification would render the prior art invention being modified unsatisfactory for its intended purpose, then there is no suggestion or motivation to make the proposed modification.") (citations omitted). In

point of fact, the requirement for the immune antisera *teaches away* from using other sources of antibodies for selection.

Similarly, the modification of Granoff to use the phage library of antibody fragments of de Kruif renders Granoff's invention unsatisfactory for its intended purpose. Granoff is directed to methods of identifying chemical compounds that mimic the native antigens of *Neisseria meningitidis* and can be used in a vaccine composition without eliciting autoreactive antibodies. *See, e.g.,* Granoff at Abstract. Granoff seeks to identify these "molecular mimetics" for *N. meningitidis* by employing, *inter alia*, the identification of immunodominant epitopes using methods such as Geysen, *i.e.*, using immune antisera. *See* Geysen at col. 10:5-13. Modifying Granoff to employ the plurality of antibody fragments of de Kruif would not help identify immunodominant epitopes that can elicit a protective immune response *in vivo* without eliciting autoreactive antibodies. Granoff limits its disclosure to the use of immunodominant epitopes (and identification of molecular mimetics of those epitopes), and the identification of epitopes not recognized by immune antisera would be both wasteful and counterproductive for Granoff's invention. Thus, to modify Granoff using the disclosure of de Kruif to result in the claimed methods renders Granoff's method completely unsuitable for its intended purpose and plainly ignores Granoff's teaching away from the use of source other than immune antisera for epitope selection.

The lack of motivation to combine the cited references is further evidenced in the Declaration of John de Kruif pursuant to 37 C.F.R. § 1.132 submitted herewith as Exhibit E. According to Dr. de Kruif, the de Kruif reference contains no teaching or suggestion of using an oligopeptide library. *See* Exhibit E at ¶4. Dr. de Kruif attests to Geysen's teachings as limited to the identification of immunodominant epitopes and lacking the ability to identify novel antibody nucleic acid sequences. *Id.* He further indicates that the modification of Geysen using the teachings of de Kruif as suggested by the Examiner results in the loss of Geysen's primary function, *i.e.*, to identify immunodominant epitopes. *Id.* According to Dr. de Kruif, this would not provide one of ordinary skill in the art with the motivation to combine these references. Dr. de Kruif also notes that Granoff is similarly limited to the identification of immunodominant epitopes, and thus does not provide the motivation to combine the disclosure of de Kruif with Granoff.

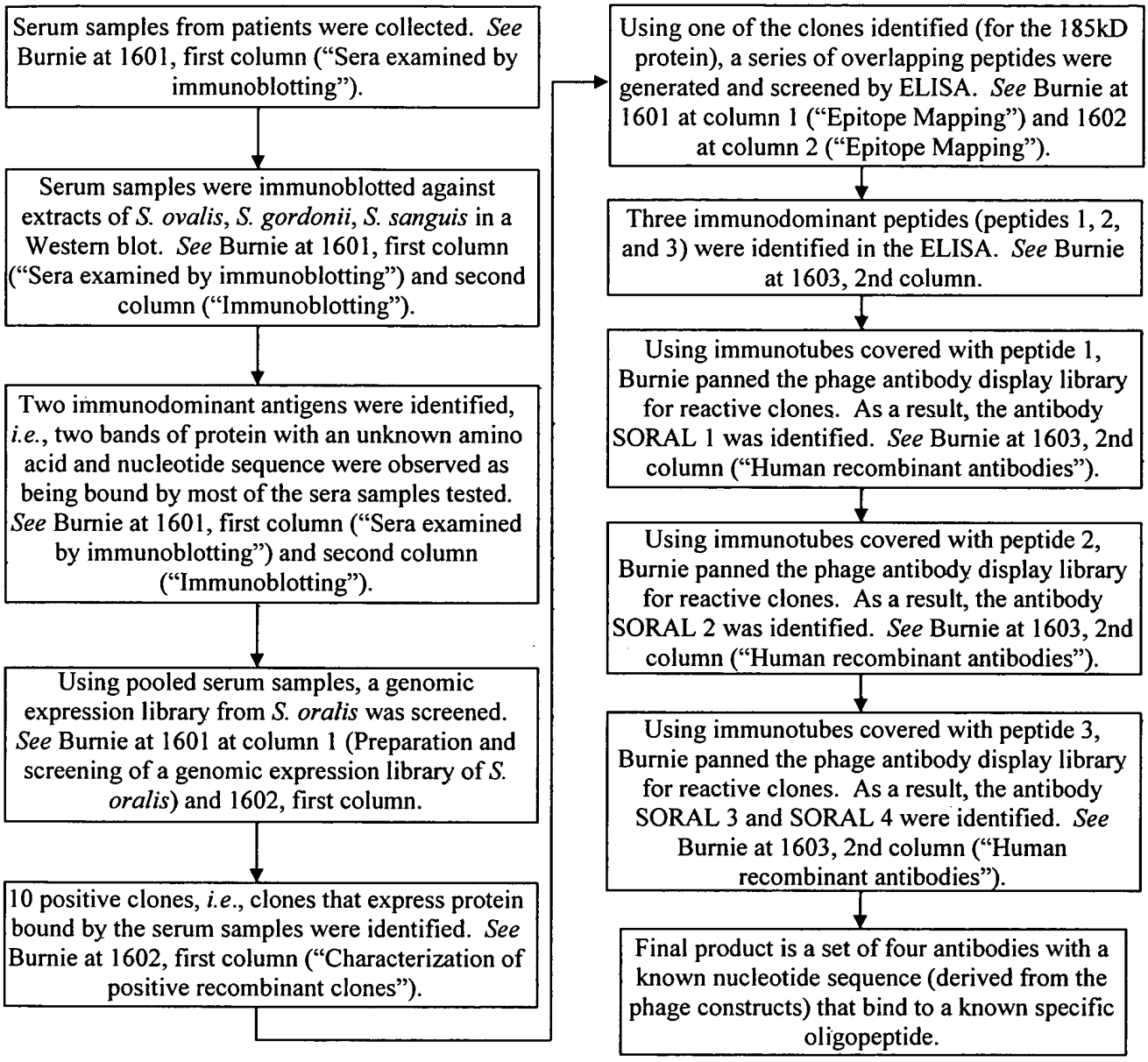
Applicants respectfully submit there is no motivation to combine the teachings of de Kruif with Geysen or Granoff to result in the claimed methods because such a combination destroys the intended purpose of Geysen and Granoff. Further, the "mere fact that reference can be combined or modified does not render the resultant combination obvious unless the prior art also suggests the desirability of the combination." See MPEP § 2143.01 (citations omitted) (emphasis added). de Kruif method's readily achieves the disclosed objective: to identify new antibodies to a variety of whole antigens or large antigen domains, and without more lacks any suggestion to combine its teachings with that of Geysen and Granoff. The rejection appears to based on nothing more than an impermissible hindsight reconstruction based upon the instant specification. Applicants respectfully request that if the Examiner's rejection is based on facts within his personal knowledge, the Examiner will support this rejection with those facts in an affidavit by the Examiner according to MPEP § 2144.03, which states:

> When a rejection is based on facts within the personal knowledge of the examiner, the data should be stated as specifically as possible, and the facts must be supported, when called for by the applicant, by an affidavit from the examiner.

Burnie

Claims 1, 3, 5-10, and 13-18 were rejected under 35 U.S.C. § 103 (a) as being unpatentable over Burnie et al., *Infection Immunity* 64:1600-08 (1996) for reasons of record. Applicants traverse this rejection.

Applicants respectfully submit that Burnie fails to teach or suggest each and every element of the claimed methods and therefore fails to anticipate the claimed methods as discussed in previous responses. More specifically, Burnie discloses the following method:



As illustrated above, the method disclosed by Burnie is complex and laborious requiring multiple steps of selection and more than one source of antibodies for the selection process. In contrast, the claimed methods are straightforward and simple, conferring the ability to isolate the nucleotide sequence to an antibody or antigen-binding fragment thereof specific for a discreet peptide on a large scale. The claimed methods lack any requirement for immune antisera and is not limited by the identification of immunodominant epitopes. In other words, Applicants successfully

omit numerous steps in Burnie's method to achieve a more efficient and ultimately more powerful method for the isolation of the nucleotide sequences of antibodies specific for a particular peptide sequence. *See* MPEP 2144.04(II)(B) ("Note that the omission of an element and retention of its function is an indicia of unobviousness.") (citations omitted). According to Dr. de Kruif, the method of Burnie is distinct in many features, encompasses many additional steps, and multiple sources of antibodies are required. *See* Exhibit E at ¶6. Therefore, Burnie fails to render the claimed methods *prima facie* obvious.

For at least these reasons, the rejections under 35 U.S.C. § 103 (a) may be withdrawn.

CONCLUSION

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to withdraw the outstanding rejections of the claims and to pass this application to issue.

In the event the U.S. Patent and Trademark office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to Deposit Account No. 03-1952 referencing docket no. 313632000600. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Dated: January 7, 2005

Respectfully submitted,

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Identification of epitopes within a protein region.

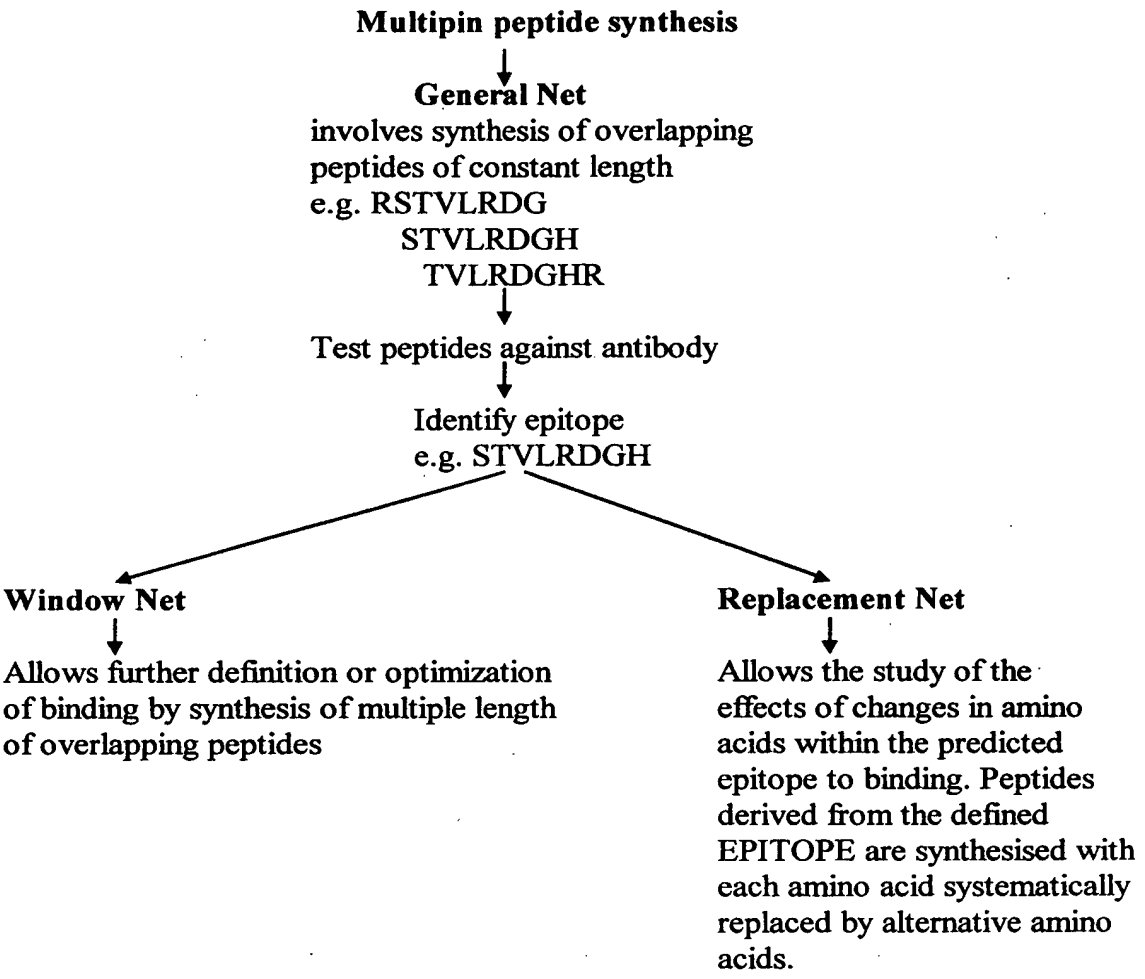

Figure 6 Identification of epitopes within a protein region.

7 Applications of Pepscan

Pepscan has been used in a wide range of applications from the study of antigen–antibody interactions (18, 22) to identification of epitopes on monoclonal antibodies (23–25) and in the study of autoantibodies (19, 26), to detection of epitopes on toxins (20, 21), viruses (27–34), bacteria (35–39), parasites (40), tumour suppressor protein (41), enzymes (42, 43), hormones (44, 45), in the design of vaccines (46–52), and even used for the location of the epitopes/sweet taste determinants (active site) of sweet tasting protein thaumatin (53). Rodda (18) studied the antibody response to myoglobin using Pepscan. A hexapeptide epitope was identified and detailed analysis of the epitope carried out by replacement net analysis showed the N-terminal leucine to be crucial to the binding of antibody to the peptide.

Using the Pepscan method, Koshy *et al.* (51) studied the binding of monoclonal antibody to chicken riboflavin carrier protein (RCP). A novel neutralization site

conserved in all known bovine respiratory syncytial virus (BRSV) and human RSV strains was identified by Pepscan analysis of monoclonal antibodies raised against fusion protein complex of BRSV. This linear conserved epitope may be a potential candidate for a peptide based vaccine which can produce neutralizing antibodies to all groups and subgroups of RSV (52).

Cross-reactive idiotypes have been detected on anti-DNA autoantibodies in SLE. Autoantibodies associated with SLE include those that bind to SmB/B' peptides. Overlapping octapeptides of SmB/B' were tested by Pepscan analysis against sera from normal and SLE patients and an octapeptide sequence identified (18). Using a set of overlapping peptides representing the VH and VL regions of monoclonal antibodies derived from mouse models of SLE and reacting these against normal and lupus mice sera, a mosaic of antibody V-region idiotypes were identified (26).

Pepscan has been used to identify a major B cell epitope within the immunodominant nucleoprotein amino subregion of the hepatitis C virus nucleocapsid protein (23).

Epitope mapping of scorpion neurotoxin II from *Androctonus australis* by the Pepscan method identified a new antigenic region for AaH II antibodies, fine analysis showed residues lysine, aspartic acid, and glycine to be important in binding (20).

Using the Pepscan method, the binding of α-bungarotoxin to Torpedo acetylcholine receptor was studied. The epitopes of 12 MCAs against the cytoplasmic side of acetylcholine receptor (AChR) α-subunit were mapped using 300 sequential peptides, a very immunogenic cytoplasmic epitope (VICE-α) was identified (25).

Epitope characterization of monoclonal antibodies against mucin protein core identified a short hydrophilic region in the MUC 1 mucin core as immunodominant in the induction of antibodies (54).

Epitope mapping of a novel fimbrial protein, Pg II from *Porphyromonas gingivalis* revealed seven immunodominant regions which reacted with sera from patients with periodontal diseases (39).

Interesting information was obtained by Pepscan analysis of horseradish peroxidase (HRP isoenzyme C). Epitopes detected in loops and folds of the HRP peptide chain with irregular shapes contained functionally important residues like Arg-38 of the active site of the enzyme and Phe-142 and 143 which form a channel allowing aromatic residues to reach the active site while amino acid residues which formed calcium binding sites did not form part of the epitope (43).

An amino acid sequence (PLITHVLPFEKINE) from alcohol dehydrogenase was studied and a pentamer (HVLPF) binding to a monoclonal antibody identified (42). Potential vaccine candidates have been elucidated by Pepscan studies on the glycoprotein E of *Varicella zoster* virus (47).

Pepscan has also provided the methodology for the analysis of humoral responses induced by vaccination (48, 49) and the study of the fine-specificity of antibodies induced by HIV candidate vaccines (50). Pepscan provides a methodology for the design, evaluation, and selection of candidate vaccines.

8 Other systems for epitope analysis

8.1 Simple precision original test system (SPOTs)

Another method for multiple peptide synthesis and epitope analysis is SPOTs (14) available from Genoysis. In this system, up to 96 peptides are synthesized in parallel using the Fmoc chemistry on a derivatized membrane (instead of pins) and the membrane probed with antibody. The SPOTs has an 8 × 12 cm format with 96 spots allowing 96 peptides to be simultaneously synthesized (one on each spot). The membrane is pre-activated ready for the first amino acid to be added, coupling takes 15–30 minutes, after deprotection the second amino acid is added, each complete cycle taking 90 minutes. Up to a maximum of 15 cycles can be performed.

The membrane with the completed peptides can be probed with antibody. The membrane is blocked, incubated with test antibody, washed, and incubated with species-specific enzyme-conjugated secondary antibody, followed by a wash, and finally incubated with enzyme substrate. Peptide epitopes recognized by the antibody light up as spots on the membrane. As with the pins, the membrane can be cleaned and re-probed allowing for optimization of antibody dilutions. In the SPOTs the peptides can also be cleaved off the membrane, lyophilized, and used in different assays. The coupling efficiencies of three different coupling methods have also been studied by Molina *et al.* (55).

8.2 Tea-bag synthesis

An alternative to Pepscan is the use of T bag synthesis where multiple peptides are simultaneously synthesized on resin (5) or on paper discs (7) as the solid phase contained in separate solvent permeable packets similar to tea-bags. Various methods for multiple peptide synthesis on solid phase have been described. Houghten's T bag synthesis (5) method followed shortly after the peptide synthesis method on pins, this was then followed by synthesis on paper (7, 56), cotton (57–59). In 1990, Krachnak *et al.* (60) described solid phase multiple peptide synthesis on paper discs followed by determination of antibody binding to the disc-bound peptides.

8.2.1 'T' bag (tea-bag) synthesis using resin

In the 'T' bag synthesis method described by Houghten (5), resin is used as the solid phase. The method for peptide synthesis is essentially that described by Merrifield (2) and all resins commonly used for SPPS can be employed.

50–100 mg (0.2–0.8 meq/g) of standard Boc amino acid resin is placed into 15 × 20 mm polypropylene (mesh size 74 μm) pouches. Each of the bags is numbered inside with indelible black ink and the bag permanently sealed. This ensures that each bag is easily identifiable. The bags are now ready for use for simultaneous synthesis of a large number of different peptides as well as for concurrent synthesis of multiple analogues of an individual peptide. More than 100 peptides can be synthesized at a time with quantities of more than 10 mg each.

All the bags are washed, deprotected, and neutralized in a reaction vessel. The bags are then taken out and reacted with individual solutions of protected amino acids for coupling. All the bags requiring a certain amino acid can be processed in the same vessel. After coupling, the bags are put in the reaction vessel for washing, deprotection, and neutralization ready for the addition of the next amino acid. Additional cycles of the washing, deprotection, neutralization, and coupling are carried out until the synthesis is complete. Any variations, e.g. single residue replacement/omissions, shorter chain length can be carried out by removing the bag at the appropriate step completion, modified separately, and added back to the reaction vessel for completion.

After completion of synthesis, protected peptide resins still within the bags are cleaved and the peptides extracted from the resins. The average purity of the peptides obtained was 84% (70–94%). The purity of the peptides synthesized by this method was found to be good or better than the purity of peptides prepared by free resin synthesis.

Using this method a series of 247 replacement analogues of an antigenic peptide sequence (aa 98–110) of influenza HA1 and 13 controls were synthesized (in two weeks). These peptides were probed with monoclonal antibody using a standard ELISA to determine the importance of individual amino acids in antibody binding. This 'T' bag synthesis approach has been used for synthesizing synthetic peptide combinatorial libraries (61, 62) using MBHA resin, t-Boc chemistry, and polypropylene mesh pouches (see Section 8.3.2).

8.2.2 T bag synthesis using paper discs

In this method (7) peptides were simultaneously synthesized on paper discs using Fmoc chemistry. Discs of filter paper (Schleicher and Schull) with 0.6 cm diameter were punched out, activated, and derivatized to get free amino groups on them. 100 of these derivatized discs (200 mg) were then placed in T bags made of 75 μm polypropylene mesh, the bags indelibly marked, and sealed. N-terminal Fmoc protected and side chain protected amino acid was then coupled on the disc, followed by side chain deprotection and washing. Cycles of coupling, deprotection, and washing were carried out until the synthesis was complete. Finally side chain deprotection was carried out. The peptides were ready to be tested for epitope mapping in solid phase immunological procedures without detachment from the paper discs. This method allows the simultaneous synthesis and subsequent immunological testing of large numbers of peptides.

Using this method, epitope mapping was carried out on the feline major allergen Fel d I. A total of 15 000 paper disc-bound peptides (146 nonapeptides overlapping by 8 amino acid residues) were synthesized simultaneously with 100 discs per bag, thereby permitting 100 epitope mapping tests. The tests were carried out using radioimmunoassay and the method was compared to Pepscan where the same peptides were synthesized and binding of each serum tested on pins. Results of paper disc RIA and pin ELISA showed binding of peptides 41–49 to 45–53 and peptides 43–51 and 44–52 in chain 2 of Fel d I respectively.

Studies on the structural requirement for ligand binding to the neuropeptide Y receptor from rat cerebral cortex have been carried out using analogues of the neuropeptide Y model peptide synthesized by the 'T' bag method (63). The T bag peptide synthesis approach using Fmoc chemistry and simultaneous multiple peptide synthesis has been used for antigenic mapping of viral proteins (64). The role of individual amino acids in binding human and macaque antibodies was determined in the human immunodeficiency virus type 1 (HIV-1) gp 41, residues 594–613. Using decapeptides with 9 amino acid overlap, amino acids 599–603 were found to be the main recognition site for 19 human anti-HIV positive sera.

8.3 Peptide epitope libraries

Epitope libraries provide a method for identifying peptide ligands for antibodies, receptors, or other binding proteins and provide a tool to rapidly identify lead ligands in the drug discovery process (65).

An alternative to mapping epitopes via the antigen is to employ combinatorial methods whereby a library of peptides is generated. The library of peptides can be generated by chemical synthesis (1, 61, 62, 66, 67) or biologically by phage display systems (68). Only chemically synthesized peptide libraries will be dealt with here. The library is a vast collection of all theoretically possible peptides consisting of 4, 5, 6, and so on amino acid residues where the total number of variations is calculated as 20^n, n being the number of residues and 20 is the number of optional amino acids. Therefore to synthesize a library of pentapeptides, the total number of variants (i.e. complexity of the library) would be $20^5 = 3.2 \times 10^6$ peptides whereas for a library of hexapeptides the total number of peptides would be $20^6 = 6.4 \times 10^7$ (69).

8.3.1 Peptide libraries on pins

Geysen *et al.* (22, 70), using replacement net analysis showed that for significant binding to an antibody, three amino acid residues within the peptide sequence should have both the correct identity and position and at least two of the three amino acids should be adjacent to one another. If two amino acid positions are fixed, 400 peptide mixtures would be required to synthesize all possible hexapeptides. These 400 hexapeptide mixtures are assayed for binding by a monoclonal antibody. The best binding peptide mixture is identified and subsequent re-synthesis and screening of the peptide mixture with first pair of amino acids extended to three defined residues, followed by four, and so on. Geysen *et al.* (71) using this strategy concluded that what is important is that the peptide should have complementarity between antigen binding site and the surface of the antibody with respect to shape and charge. This led to the term 'Mimotope' which is defined as the optimum binding peptide that mimics the binding of the epitope without necessarily bearing any relationship to the primary sequence. Elucidation of such a binding peptide can be achieved by:

(a) Synthesizing peptide mixtures, e.g. libraries as described above or in Section 8.3.2.

(b) Starting with a dipeptide and building a defined peptide on it, the 'dipeptide strategy' (Section 8.3.3).

8.3.2 Combinatorial libraries using 'T' bags

Houghten *et al.* (61) have developed synthetic peptide combinatorial libraries (which uses an iterative selection and enhancement process to define the most active sequence) composed of free peptides in quantities which can be used in virtually all existing assays). This method essentially uses the same principle as the Geysen *et al.* (71) library approach except that the synthesis is carried out in T bags as described in Section 8.2.1 and the peptides can be used in solution. There is no limitation to the number of peptides that can be synthesized. A hexapeptide library (with N-terminal acetylation and C-terminal amidation) was synthesized, starting with the first two amino acids positions defined in each peptide and the last four positions of equimolar ratios of 18 of the 20 natural L-amino acids (cysteine and tryptophan were left out in the initial library for ease of synthesis). The initial starting sequence can be represented as Ac-O_1O_2XXXX-NH_2, where O_1 and O_2 are the defined amino acids and X is the equimolar mixture of the 18 amino acids used, hence 324 hexapeptides mixtures were synthesized in the first round. In this method the assay used for testing was the ability of the peptide mixtures to inhibit of binding of a monoclonal antibody to a 13-residue peptide. The optimum defined amino acid mixture that gave maximum inhibition was noted and new peptide mixtures were synthesized with the third amino acid defined (at this stage tryptophan was included in the X position). The iterative process was carried out for the three remaining positions. Using this method a library composed of over 34 million hexapeptides was synthesized and used to identify an antigenic determinant of a monoclonal antibody. The same approach was used in the development of new antimicrobial peptides. A conceptually different approach is the positional scanning synthetic peptide combinatorial library (PS-SPCL) (62) where ten positional decapeptide libraries were synthesized. Each of the ten decapeptide positional peptide libraries is made up of 20 peptide mixtures with a **single** amino acid position defined and the other 9 positions composed of the 18 amino acid mix as described above.

8.3.3 Dipeptide net

In this method, all possible dipeptides are synthesized, each on a separate support, and screened against the antibody. The sequence of the dipeptide that binds best is then extended by re-synthesizing 20 analogues of the dipeptide with only one of the 20 amino acids for the third residue, then taking the best of the tripeptide and synthesizing its analogues with one of the 20 amino acids as the fourth residue and so on. This eventually leads to the identification of an optimum binding peptide. There is a possibility with this approach that a low binding dipeptide which is discarded might actually be as a high binder in a longer peptide.

8.3.4 Peptide libraries on beads

Lam *et al.* (72) have described a method for the synthesis of a peptide library consisting of a set of all possible peptides on resin beads using a 'one-bead, one-peptide' approach. This method involves the synthesis of a large library comprising millions of beads, each bead containing a single representing the universe of possible random peptides in roughly equimolar ratios. Different amino acids have different coupling rates and therefore use of a random mixture of amino acids in a peptide synthesis protocol would lead to unequal representation. To circumvent this, a 'split synthesis' approach was used. A pool of resin beads was distributed into separate reaction vessels containing a single amino acid. This first amino acid was coupled to the resin and after the first coupling cycle, the resins were pooled, split, and distributed into reaction vessels. The process of coupling the second amino acid was carried out. This randomizing and splitting process was continued till the required length of peptides was reached. Using this method, each bead should only contain a single peptide species. Standard solid phase Boc and Fmoc chemistries are applied.

For tripeptides starting with three reaction vessels and three cycles of coupling, 27 tripeptides each with a different sequence would be produced. On a larger scale, starting with 19 reaction vessels and synthesis of pentapeptides, would produce a library of up to 19^5, i.e. 2 476 099 individual peptides of differing sequence. Such a synthesis would take a few days.

Screening of the peptide library, which may seem a daunting task was quite cleverly achieved. The peptide beads were put in a Petri dish and reacted with enzyme labelled or fluorescein labelled acceptor molecule. Where there was binding of the acceptor to the peptide bead, staining was visible which could be easily visualized under a low power microscope. The reactive beads were then removed and microsequenced after removal of the acceptor molecule. Screening of a library of several million beads could be accomplished in an afternoon using 10–15 Petri dishes.

This method has been used to study the binding of a monoclonal antibody to β endorphin specific for a pentapeptide YGGFL. Six reactive beads were retrieved from two million beads screened from the pentapeptide library, the affinities of the ligands were better than those obtained by a phage library method. Using the same pentapeptide library, the peptides binding sequence for streptavidin was found to be have a consensus sequence of HPQ (72).

This method of synthesis and screening of peptide libraries is simple and identifies ligands with affinities virtually identical to the natural ligand. Both D- and L-amino acids can be used and most importantly the peptides sequences on the beads do not need to be predetermined as only those beads that light up need to be sequenced.

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Review

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The production of antibody fragments and antibody fusion proteins by yeasts and filamentous fungi

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Abstract

In this review we will focus on the current status and views concerning the production of antibody fragments and antibody fusion proteins by yeasts and filamentous fungi. We will focus on single-chain antibody fragment production (scFv and V_{HH}) by these lower eukaryotes and the possible applications of these proteins. Also the coupling of fragments to relevant enzymes or other components will be discussed. As an example of the fusion protein strategy, the 'magic bullet' approach for industrial applications, will be highlighted.

Introduction

Antibodies (also called immunoglobulins) are glycoproteins, which specifically recognise foreign molecules. These recognised foreign molecules are called antigens. When antigens invade humans or animals, an immunological response is triggered which involves the production of antibodies by B-lymphocytes. By this immunological response, microorganisms, larger parasites, viruses and bacterial toxins can be rendered harmless. The unique ability of antibodies to specifically recognise and bind with high affinity to virtually any type of antigen, made them interesting molecules for medical and scientific research.

In 1975 Köhler and Milstein developed the monoclonal antibody technology [1] by immortalising mouse cell lines that secreted only one single type of antibody with unique antigen specificity, called monoclonal antibodies (mAbs). With this technology, isolation and production of mAbs against protein, carbohydrate, nucleic acids and hapten antigens was achieved. The technology resulted in a rapid development of the use of antibodies in diagnostics (*e.g.* pregnancy tests; [2]), human therapeutics and as fundamental research tools.

More applications outside research and medicine can be considered, such as consumer applications. Examples are the use of antibodies in shampoos to prevent the formation of dandruff [3] or in toothpaste to protect against tooth decay caused by caries [4]. For these purposes large quantities of antibodies are required. However, for these applications on a larger scale there were some major problems concerning the expensive production system based on mammalian expression, the difficulty of producing antibodies in bulk amounts and the low stability and solubility of some antibodies under specific (harsh) conditions.

In this review we will discuss the possibilities of large-scale production of antibodies and fragments thereof by relevant expression systems. Requirements are that the system used for production is cheap, accessible for genetic modifications, easily scaled up for greater demands and safe for use in consumer applications.

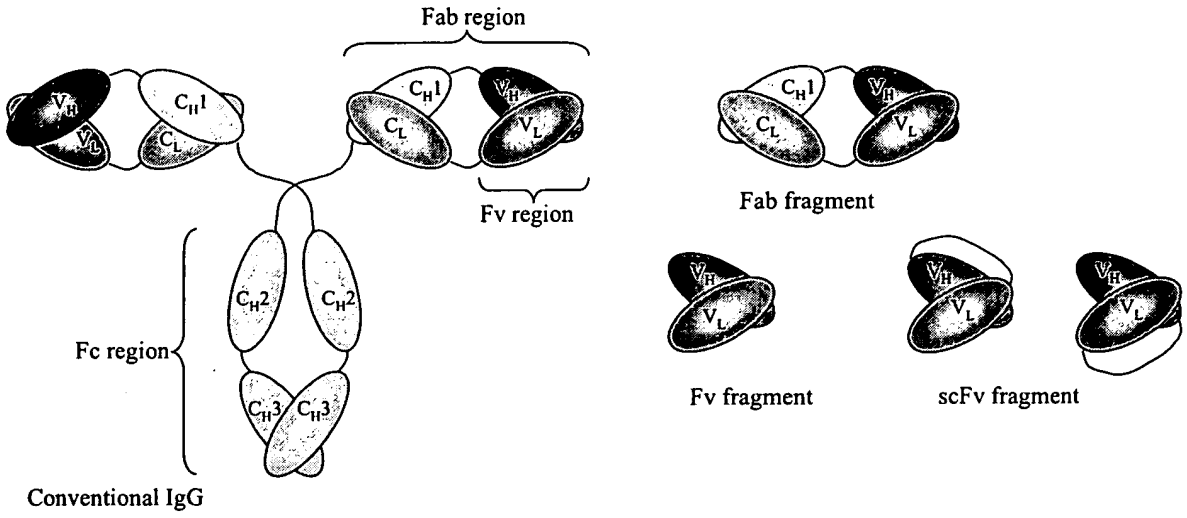

Figure 1
Schematical representation of the structure of a conventional IgG and fragments that can be generated thereof. The constant heavy-chain domains C_H1, C_H2 and C_H3 are shown in yellow, the constant light-chain domain (C_L) in green and the variable heavy-chain (V_H) or light-chain (V_L) domains in red and orange, respectively. The antigen binding domains of a conventional antibody are Fabs and Fv fragments. Fab fragments can be generated by papain digestion. Fvs are the smallest fragments with an intact antigen-binding domain. They can be generated by enzymatic approaches or expression of the relevant gene fragments (the recombinant version). In the recombinant single-chain Fv fragment, the variable domains are joined by a peptide linker. Both possible configurations of the variable domains are shown, i.e. the carboxyl terminus of V_H fused to the N-terminus of V_L and vice versa.

First, structure and characteristics of antibodies and antibody fragments generated thereof will be discussed, followed by the impact of recombinant DNA technology and antibody engineering techniques on the generation and modification of antibodies and antibody fragments. The modification of antibodies is of major interest since changes in their functionality and physico-chemical properties will broaden their application area. For most applications only the antigen-binding site of the native antibody molecule is required and even preferred. By the development of recombinant DNA technology and the increasing knowledge on the structure of antibody molecules created the opportunity to clone and engineer smaller fragments of antibody genes [5,6] and subsequent alter their functions, for example improve the affinity for their antigen. Besides that, recombinant DNA technology provides the possibility to generate fusion proteins or 'Magic bullets', consisting of an antibody fragment fused to an effector molecule.

In this review the various expression systems for these type of protein will be outlined. We will detail on using yeasts and filamentous fungi as suitable expression systems for antibody fragments and antibody fusion proteins.

Antibodies and their unique antigen binding domains

Whole antibodies

In vertebrates five immunoglobulin classes are described (IgG, IgM, IgA, IgD and IgE), which differ in their function in the immune system. IgGs are the most abundant immunoglobulins in the blood and these molecules have a molecular weight of approximately 160 kDa. They have a basic structure of two identical heavy (H) chain polypeptides and two identical light (L) chain polypeptides (Figure 1). The H and L chains, which are all β-barrels, are kept together by disulfide bridges and non-covalent bonds (for a review about antibody structure see [7]). The chains themselves can be divided in variable and constant domains. The variable domains of the heavy and light chain (V_H and V_L) which are extremely variable in amino acid sequences are located at the N-terminal part of the antibody molecule. V_H and V_L together form the unique antigen-recognition site. The amino acid sequences of the

remaining C-terminal domains are much less variable and are called C_H1, C_H2, C_H3 and C_L.

Fc fragment

The non-antigen binding part of an antibody molecule, the constant domain Fc mediates several immunological functions, such as binding to receptors on target cells and complement fixation (triggering effector functions that eliminate the antigen). The Fc domain is not essential for most biotechnical applications, relying on antigen binding. The Fc fragment, which is glycosylated, can have different effector functions in the different classes of immunoglobulins.

Antigen binding region

The unique antigen-binding site of an antibody consists of the heavy and light chain variable domains (V_H and V_L). Each domain contains four conserved framework regions (FR) and three regions called CDRs (complementarity determining regions) or hypervariable regions. The CDRs strongly vary in sequence and determine the specificity of the antibody. V_L and V_H domains together form a binding site, which binds a specific antigen.

Antibody fragments generated thereof

Several functional antigen-binding antibody fragments could be engineered by proteolysis of antibodies (papain digestion, pepsin digestions or other enzymatic approaches), yielding Fab, Fv or single domains (Figure 1).

Fab fragments

Fab fragments (fragment antigen binding) are the antigen-binding domains of an antibody molecule, containing V_H + C_H1 and C_L + V_L. Between C_L and C_H1 an interchain disulfide bond is present. The molecular weight of the heterodimer is usually around 50 kDa [8]. Fab fragments can be prepared by papain digestions of whole antibodies.

Fv fragments

The minimal fragment (~30 kDa) that still contains the whole antigen-binding site of a whole IgG antibody is composed of both the variable heavy chain (V_H) and variable light chain (V_L) domains. This heterodimer, called Fv fragment (for fragment variable) is still capable of binding the antigen [9]. Normally, native Fv fragments are unstable since the non-covalently associated V_L and V_H domains tend to dissociate from one another at low protein concentrations.

Single domains

Single domain antigen binding fragments (dAbs) or V_Hs were generated in the past [10,11]. They have good antigen-binding affinities, but exposure of the hydrophobic surface of the V_H to the solvent, which normally interacts with the V_L, causes a sticky behaviour of the isolated V_Hs. It turned out to be difficult to produce them in soluble form, although replacement of certain amino acids increased solubility of these single domains (see also **Llama Heavy-chain antibody fragments**) Besides that, their affinity for the antigen was much less compared with other antibody fragments [12].

***Heavy-chain antibodies in* Camelidae**

In 1993 Hamers-Casterman *et al.* [13] discovered a novel class of IgG antibodies in *Camelidae* (camels, dromedaries and llamas). These antibodies are devoid of light chains and therefore called 'heavy-chain' IgGs or HCAb (for heavy-chain antibody; Figure 2). HCAbs have a molecular weight of ~95 kDa instead of the ~160 kDa for conventional IgG antibodies. Their binding domains consist only of the heavy-chain variable domains, referred to as V_{HH}s [14] to distinguish it from conventional V_Hs. Since the first constant domain (C_H1) is absent (spliced out during mRNA processing due to loss of a splice consensus signal; [15,16]), the variable domain (V_{HH}) is immediately followed by the hinge region, the C_H2 and the C_H3 domains. Although the HCAbs are devoid of light chains, they have an authentic antigen-binding repertoire. The current knowledge about the genetic generation mechanism of HCAbs is reviewed by Nguyen *et al.* [17,18].

Recombinant antibodies, antibody fragments and antibody fusion proteins

The development and applications of recombinant DNA technology led to the design of several new antibodies and antibody fragments. Firstly, functionalities of these proteins may be altered resulting in novel and improved functions. One of the possible applications of recombinant whole antibodies is the use in human therapeutics (see also **Recombinant whole antibodies**). Secondly, smaller antibody fragments may be synthesised having the advantage over whole antibodies in applications requiring tissue penetration and rapid clearance from the blood or kidney. Moreover, the use of recombinant expression systems could also be the solution for large-scale production of antibody (fragments).

Recombinant whole antibodies

The development of human(ised) antibody molecules is mostly aimed at reduction of unwanted immunological properties in medical applications [19]. Repeated doses of foreign (murine) antibody molecules could lead to an immune response in patients recognising the mouse antibody as foreign. This so-called HAMA (human anti-mouse antibody) response can lead to severe health problems.

Two strategies are developed to reduce the antigenicity of therapeutic antibodies (see also [20]). One of these strategies is chimerisation. In this case the constant murine do-

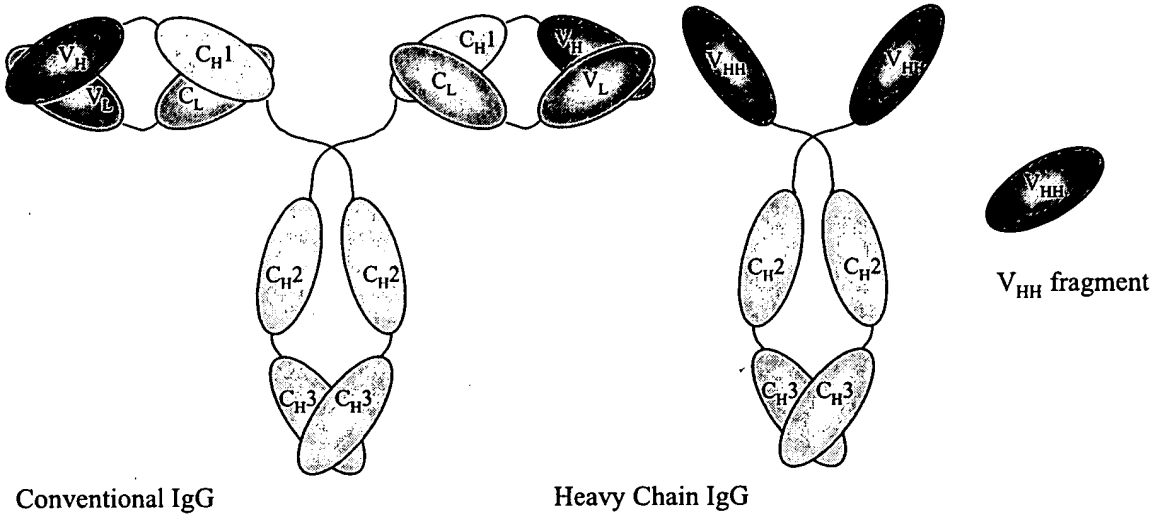

Figure 2
Schematical representation of the structure of a conventional IgG, a heavy-chain IgG antibody and the variable heavy-chain antibody fragment (V_{HH}) that can be generated of the latter. Heavy-chain antibodies found in llama and camel are only composed of heavy-chains and lack the light chain completely, as shown in this Figure. The antigen-binding domain consists of only the V_H domain, which is referred to as V_{HH} (variable heavy-chain antibody fragment), to distinguish it from a normal V_H. The constant heavy-chain domains C_H1, C_H2 and C_H3 are shown in yellow, the constant light-chain domain (C_L) in green and the variable heavy-chain (V_H or V_{HH}) or light-chain (V_L) domains in red and orange, respectively.

mains are replaced by human constant domains [21,22]. The second strategy is grafting of only the murine CDRs onto existing human antibody framework regions, which is called humanisation [22].

At present there are more than 10 recombinant antibodies approved by the US Food and Drug Administration (FDA) for use in medicine and many more are in a late stage of clinical trials. FDA approved recombinant mAbs are *e.g.* Herceptin™ (Genetech, San Francisco, CA), which targets and blocks the growth factor Her2 on the surface of breast cancer cells and Rituxan™ (IDEC Pharmaceuticals Inc., San Diego, CA) used against non-Hodgkin's lymphoma (see for more examples [23,24]). The use of recombinant antibodies for medical purposes does not require a cheap large-scale production process *per se*, since only a limited amount of pure preparations is needed.

Production of recombinant antibody fragments by Escherichia coli

Much work on antibody fragment production has been focussed on *Escherichia coli* as an expression system (reviewed in [25]). The advantage of this system is the ability to produce proteins in relative large amounts. Besides that, *E. coli* is easily accessible for genetic modifications, requires simple inexpensive media for rapid growth and they can easily be cultured in fermentors permitting large-scale production of proteins of interest. Several antibody fragments have been produced in functional form (*e.g.* [8,9,26,27]) and expression of relevant gene segments also permitted the production of the recombinant antibody fragments. The problem of stability has been tackled by generation of single-chain Fv (scFv) or disulfide stabilised Fv (dsFv) fragments.

Selection of antibody fragments with improved functionalities

In 1990 McCafferty *et al.* [28] showed that antibody fragments could be displayed on the surface of filamentous

phages, called phage-display. This technology is based on the fusion of the antibody variable genes to a phage coat protein gene (*e.g.* [20]). After displaying an antibody fragment on the protein surface of the phage, antigen specific phages can be selected and enriched by multiple rounds of affinity panning (*e.g.* reviewed in [29,30]). This technique makes it possible to select phages that bind almost any antigen, including those previously considered to be difficult, such as self-antigens or cell surface proteins.

Libraries can be prepared from variable genes isolated from immunised animals, non-immunised sources (naïve libraries, thus avoiding the need for immunisation) or even (semi-) synthetic libraries can be constructed. The V genes can be subjected to random mutagenesis, chain or DNA shuffling methods [31], mimicking the natural hypermutation mechanism.

Single-chain Fv fragments and multimers

An attractive recombinant antibody fragment is the single-chain Fv (scFv) fragment (reviewed in [32,33]). It has a high affinity for its antigen and can be expressed in a variety of hosts [34]. These and other properties make scFv fragments not only applicable in medicine (reviewed in [35]), but also of potential for biotechnological applications. In the scFv fragment the V_H and V_L domains are joined with a hydrophilic and flexible peptide linker, which improves expression and folding efficiency [36,37]. Usually linkers of about 15 amino acids are used, of which the $(Gly_4Ser)_3$ linker has been used most frequently [35]. Unfortunately, some scFv molecules have a reduced affinity compared to the parental whole antibody or Fab molecule [12,38,39]. Besides that, scFv molecules can be easily proteolytically degraded, depending on the linker used [40]. With the development of genetic engineering techniques these limitations could be practically overcome by research focussed on improvement of function and stability, as discussed in [32]. An example is the generation of disulfide-stabilised Fv fragments where the V_H-V_L dimer is stabilised by an interchain disulfide bond [38,41,42]. Cysteïnes are introduced at the interface between the V_L and V_H domains, forming a disulfide bridge, which holds the two domains together (reviewed in [43]).

Dissociation of scFvs results in monomeric scFvs, which can be complexed into dimers (diabodies), trimers (triabodies) or larger aggregates ([44], reviewed in [45]). The simplest designs are diabodies that have two functional antigen-binding domains that can be either similar (bivalent diabodies) or have specificity for distinct antigens (bispecific diabodies). These bispecific antibodies allow for example the recruitment of novel effector functions (such as cytotoxic T cells) to the target cells, which make them very useful for applications in medicine (reviewed in [46,47]).

Llama Heavy-chain antibody fragments (V_{HH}s)

The other type of interesting antibody fragments are V_{HH}s (see Figure 2) comprising the smallest available intact antigen-binding fragment (~15 kDa, 118–136 residues [48,49]). The affinities found for V_{HH}s were in the nanomolecular range and comparable with those of Fab and single chain Fv (scFv) fragments [50,51]. Besides that V_{HH}s are highly soluble and more stable than the corresponding derivatives of scFv and Fab fragments [50,52]. V_{HH}s carry amino acid substitutions that make them more hydrophilic and prevent the prolonged interaction with BiP (Immunoglobulin heavy-chain binding protein), which normally binds to the H-chain in the Endoplasmic Reticulum (ER) during folding and assembly, until it is displaced by the L-chain [53]. There are indications that this increased hydrophilicity improves secretion of the V_{HH}s from the ER. Hence, production of V_{HH}s in commercially attractive microorganisms may be favourable.

Several ways are described to obtain functional V_{HH}s: from proteolysed HCAb of an immunised camelid, direct cloning of V_{HH} genes from B-cells of an immunised camelid resulting in recombinant V_{HH}s or from naïve or synthetic libraries [49]. V_{HH}s with desired antigen specificity could be selected by phage display (see **Selection of antibody fragments with improved functionalities**). Using V_{HH}s in phage display is much simpler and more efficient as compared with Fabs or scFvs, since only one domain needs to be cloned and expressed to obtain a functional antigen-binding fragment [52,54].

As already noted before (see **Antibody fragments generated thereof**), classical V_Hs were difficult to produce in soluble form. To improve their solubility and prevent non-specific binding, residues located on the V_L side of V_Hs were replaced by 'V_{HH}-like' residues, mimicking the more soluble V_{HH} fragments. This process has been termed camelisation [55–57] and these camelised V_H fragments, particularly those based on the human framework, are expected to have significant advantages for therapeutical purposes in humans (reviewed in [58]).

Fusion proteins ('Magic bullets')

A completely new use of the binding capacity of antibody fragments is the design of a fusion approach, in which an effector protein is coupled to an antigen recognising antibody fragment. In human medicine this approach is referred to 'Magic bullet'. All kinds of molecules can be used as effector molecule only limited by the imagination. The gene encoding the effector may be directly fused to the gene of the antibody fragment of interest, resulting in novel bifunctional proteins [59]. Examples of the use of this approach will be given in the section **Antibody fragments and antibody fusion proteins for large-scale applications and consumer products.**

Table 1: Extracellular production of antibody fragments in prokaryotic expression systems.

Strains	Characteristics	Food grade	Production yields	References
Lactobacillus zeae	Gram$^+$	yes	scFv, ND* (secreted/cell-bound)	[91]
Bacillus subtilis	Gram$^+$	yes	10 to 15 mg/L scFv (secreted)	[143,144]
Streptomyces lividans	Gram$^+$	yes	1 mg/L Fv fragment (secreted)	[145,146]
Staphylococcus carnosus	Gram$^+$	yes	5–10 mg/L V_H domain (secreted)	[147]
Proteus mirabilis	Gram$^-$	no	40 to 200 mg/L scFv (secreted)	[148,149]
Escherichia coli	Gram$^-$	no	Several fragments (inclusion bodies/periplasmic space)	[25,34]

*ND = not determined

Applications of antibody fragments and antibody fusion proteins

Applications of antibody fragments in human medicine

The smaller the better

Most applications of recombinant antibody fragments are related to diagnosis and therapy in human medicine, which is especially focussed on the use of antibodies as the ideal cancer-targeting reagent (reviewed in [19,60–62]). For some clinical applications small antibody fragments have advantages over whole antibodies. The small size permits them to penetrate tissues and solid tumours more rapidly than whole antibodies [63] which recently also was shown for V_{HH}s [64]. Smaller antibody fragments have also a much faster clearance rate in the blood circulation, which leads to differences of selectivity [63]. Nowadays there are also promising pre-clinical and clinical trials with antibody fragments as diagnostic or therapeutical agents [61,65]. Another application of antibody fragments is to treat viral infections with so-called intrabodies, which are intracellular antibodies synthesised by the cell and targeted to inactivate specific proteins within the cell [66].

'Magic bullets' in medicine

The use of bi-functional molecules in medicine is aimed at delivery of a protein drug, which is only active where it is required. It thereby limits the dose of the drug, resulting in less side effects of the drug towards healthy tissue and/or less immunogenic response to the protein drug itself. Also the physical interaction between the target and the effector molecule increases the potency of the effector. Fusion proteins are ideal immuno agents for cancer diagnosis [67] and cancer therapeutics. An example is the use of cancer-specific bi-functional antibodies targeting potent cytotoxic molecules to tumour cells and subsequently eliminate these tumour cells without harming healthy cells [68].

Potential applications of V_{HH}s

Specific applications of V_{HH}s are foreseen in the following direction:

V_{HH}s as drug carriers

It is expected that V_{HH}s are also applicable in diagnosis and therapy in human medicine, especially when an economically feasible production, small size and stability are required (reviewed in [49]). Cortez-Retamozo et al. [64] recently showed that V_{HH}s specifically could be targeted to tumour cells, which together with the possibility of generation of bispecific V_{HH} constructs [69] is of major interest for cancer therapy.

V_{HH}s as delivery carriers in the brain

Antibodies and many other water soluble compounds are excluded from the brain by the blood-brain barrier (BBB), thus making treatment of brain-related disease very difficult. Recently, Muruganandam *et al.* [70] showed that V_{HH} were able to selectively bind to and transmigrate across the BBB in a human *in vitro* BBB model and partly *in vivo* in mice. This property can be exploited for the development of efficient antibody carriers suitable for delivery of macromolecules across the human BBB and subsequently for treatment of neurological diseases.

V_{HH}s as potent enzyme inhibitors

Hypervariable regions in V_{HH}s are on average longer than those of V_Hs [71,72]. The extended hypervariable regions of V_{HH}s are capable of penetrating deep into the cleft of active sites of enzymes, binding to novel epitopes that are not recognised by conventional antibodies [51,73,74]. Because of this property V_{HH}s may act as better potent enzyme inhibitors [51,75,76].

V_{HH}s in consumer products

Since llama V_{HH}s are very stable, even at high temperature, applications can be envisaged in which a high temperature step is involved (e.g. pasteurisation), without losing antigen-binding properties [50]. Recently it was shown that V_{HH}s could be used to prevent phage infection in cheese production processes [77], by recognising a structural protein of the phage, which is involved in recognition of the host Lactococcus lactis.

Table 2: Higher eukaryotic expression systems for heterologous protein production and possible advantages and disadvantages of the expression system.

Expression systems	Ease of molecular cloning	upscaling	Economic feasibility[1]	Pathogenic contaminants[2]	References[3]
Mammalian cells	+	+/-	+	+	[59,150–154]
Insect cells	++	+	+	+	[155–160]
Plants	++	+++	++	++	[92,93,161,162]
Transgenic animals*	+/-	+++	+/-	+/-	[163–166]
Yeasts	+++	+++	+++	+++	See references in section **Production of antibody fragments by lower eukaryotes.**
Filamentous fungi	+++	+++	+++	+++	[4,123,128]

+++ = excellent, ++ = good, + = sufficient, +/- = poor. * With transgenic animals in this context is mentioned the production of antibodies or antibody fragments in the milk of transgenic animals, for example rabbits, sheep, goats or cows [1] With economical feasibility is mentioned the time and cost of molecular cloning, upscaling and downstream processing (purification). [2] Pathogenic contaminants like viruses or pyrogens. [3] Articles dealing with production of antibodies, antibody fragments and antibody fusion proteins.

Antibody fragments and antibody fusion proteins for large-scale applications and consumer products

Many additional applications can be envisaged if an inexpensive and simple production system is available, yielding large amounts of antibody fragments that can be purified easily. The highly specific antigen-binding ability could be used for inactivating bacteria or specific enzymes that can cause spoilage of food. Other suggested applications are the use in biosensors, treatment of wastewater [78], industrial scale separation processes such as separation of chiral molecules [79], purification of specific components (proteins) from biological materials or the use as abzymes [80,81]. They have also been considered as components of novel consumer goods with new improved functionalities, in oral care and personal hygiene (*e.g.* in toothpaste or mouthwashes [82]). For dental applications antibody fragments can be coupled to enzymes to increase the concentration of antimicrobials like hypothiocyanate and hypohalites, for example glucose oxidase (GOX; [83]), galactose oxidase (GaOX; [84]) or lactate oxidase (LOX; [85]). Other examples are targeted bleach in laundry washing (*e.g.* detergents containing antibodies coupled to molecules that specifically remove difficult stains) or the use in shampoos where antibodies act to prevent dandruff by inhibiting growth of specific microorganisms causing this [3].

Suitable expression systems for the large-scale production of antibody fragments and antibody fusion proteins

To be able to use antibody fragments and antibody fusion proteins in these large scale applications, a suitable expression system has to be chosen. Several expression systems are available, both from prokaryotic (Table 1) and eukaryotic (Table 2) origin. Our main interest goes out to these systems that are able to economically produce large amount of proteins into the culture medium. Several of these systems can be considered as suitable (both from prokaryotic and eukaryotic origin). Hereafter several of these systems will be discussed, with an emphasis on yeast and fungal systems.

Drawbacks using E. coli as a host for antibody fragment production

As described in the section Production of recombinant antibody fragments by *Escherichia coli*, this micro-organism has shown to be a potential expression host for antibody fragments and fusion proteins. Although the general production yields in shake-flask cultures are low (several mg/L), in fermentation processes several g/L could be obtained (reviewed in [86]). There are two possibilities of antibody fragment production in *E. coli*, either by secretion of the fragments into the culture medium and/or periplasmic space (the compartment between the inner and outer membrane) or preparation of inclusion bodies with subsequent *in vitro* folding. However, both strategies have disadvantages that make the use of this prokaryote not attractive for the large-scale production of antibody fragments and antibody fusion proteins. Firstly, the secretion of folded and fully assembled fragments in the medium or periplasmic space is often accompanied with cell lysis and subsequent product loss. Secondly, 'toxicity' of the antibody sequence and concomitant plasmid loss is frequently observed, which hamper high production levels (reviewed in [25]). Thirdly, expression of the fragments in inclusion bodies, which often results in insoluble protein aggregates [87], demands laborious and cost-intensive *in vitro* refolding (denaturation and renaturation) and purification steps. Hence, the final yield of fragments is only a small percentage of the protein that was initially present in the inclusion bodies even though purification steps are nowadays facilitated by affinity

chromatography using C-terminal polypeptide tails, like poly-His_6 or FLAG [88,89]. Recently, production of soluble and functional scFv by *E. coli* could be increased by improving disulfide bond formation activity in the cytoplasm, using mutants and overexpression of disulfide-bond isomerase [90]. Finally, *E. coli* is unable to carry out eukaryotic post-translational modifications and is therefore not suitable when glycosylation of antibody fragments or more importantly the fusion proteins is required.

Alternative prokaryotic expression systems

E. coli is not the only available prokaryotic expression system, although it is rather dominant in the field. Alternative prokaryotic expression systems are available for antibody fragment production (Table 1). However, these will encounter similar limitations as *E. coli*, even though most organisms described in Table 1 secrete the investigated antibody fragment into the culture medium. A field where production of antibody fragments in prokaryotic cells could still be interesting, is in food grade organisms used for delivery passive immunisation in humans, by means of functional foods. In a recent article, Kruger *et al.* [91] reported the production of scFv antibody fragments against *Streptococcus mutans* by the Gram positive food grade bacteria *Lactobacillus zeae*. In experimental animals a decrease of *S. mutans* and reduced development of caries was observed.

Eukaryotic expression systems

Also several eukaryotic systems can be envisaged for large-scale production of antibody fragments and antibody fusion proteins (see also [34]), like mammalian cells, insect cells, plants, transgenic animals and lower eukaryotes (see Table 2).

The production of therapeutical whole antibodies is well established in mammalian cells. However, large-scale production is expensive and time-consuming.

'Plantibodies' can be produced in several plant target organs (reviewed in [92]). Roots, storage organs (seeds and tubers) and fruiting bodies can be suitable for mass oral (edible) applications (see [93] and references therein). Expression of scFv in transgenic plants has been proposed as a way to produce and store pharmaceutical antibodies [94,95] and as means to block physiological processes in the plant itself [96] or establish plant pathogen resistance [97]. Plants show several advantages as large-scale antibody production systems, like the ease and low costs of growing plants, even in large quantities. However, the generation of transgenic plants that express antibodies is a time consuming process and the downstream processing to isolate the expressed antibodies from the plant parts is relatively expensive and laborious.

Production of antibody fragments by lower eukaryotes

An attractive possibility for the cost-effective large-scale production of antibody fragments and antibody fusion proteins are yeast or fungal fermentations. Large-scale fermentation of these organisms is an established technology already used for bulk production of several other recombinant proteins and extensive knowledge is available on downstream processes. Besides that, yeasts and filamentous fungi are accessible for genetic modifications and the protein of interest may be secreted into the culture medium. In addition, some of their products have the so-called GRAS (Generally Regarded As Safe) status and they do not harbour pyrogens, toxins or viral inclusions.

Methylotrophic and other yeasts

The methylotrophic and other yeasts like *Candida boidinii, Hansenula polymorpha, Pichia methanolica* and *Pichia pastoris* are well known systems for the production of heterologous proteins (reviewed in [98]). High levels of heterologous proteins (milligram-to-gram quantities) can be obtained and scaling up to fermentation for industrial applications is possible [99–101].

Especially the *P. pastoris* system is used in several industrial-scale production processes [102]. Ridder *et al.* [103] were the first to report the expression of a scFv fragment by *P. pastoris*. From then on several papers reported about the use of *P. pastoris* for the production of recombinant antibodies and fragments thereof [104,105]. In shake-flask cultures a level of 250 mg/L scFv was obtained [106] and Freyre *et al.* [107] were able to obtain even an expression level of 1.2 g/L scFv fragment under fermentation conditions. However, Cupit *et al.* [108] also showed that the production of antibody fragments by *P. pastoris* is not always a success story.

Based on the described results the commercial recombinant antibody production by *P. pastoris* is promising. However, products currently obtained from *P. pastoris* are not regarded as GRAS, which may limit its use.

Wood *et al.* [109] were the first to report the production of mouse IgM by the baker's yeast *S. cerevisiae*, although only unassembled chains were detected in the culture medium. However, the production of Fab fragments was possible as was first shown by Horwitz *et al.* [110]. Although the obtained levels were low, functional Fab fragments were secreted in the culture medium. Davis *et al.* [111] expressed scFv antibody fragments in *Schizosaccharomyces pombe*. Studies on the scFv production in the non conventional yeasts *Yarrowia lipolytica* and *Kluyveromyces lactis* resulted in 10–20 mg/L functional and soluble anti-Ras scFv [112].

Filamentous fungi: Trichoderma reesei *and* Aspergillus spp

Filamentous fungi, in particular species from the genera *Trichoderma* and *Aspergillus* have the capacity to secrete large amounts of proteins, metabolites and organic acids into their culture medium. This property has been widely exploited by the food and beverage industries where compounds secreted by these filamentous fungal species have been used for decades. This has led to the GRAS status for some of their products. Filamentous fungi like *A. awamori*, *A. niger* and *A. oryzae* are therefore suitable organisms for the production of commercially interesting homologous and heterologous proteins [113–115]. Strategies to improve protein secretion by filamentous fungi are extensively reviewed in [116–119].

Production strains of *Trichoderma reesei* (*Hypocrea jecorina*) have an exceptional secretion capacity up to 35 g protein/L, where half of the secreted protein consists of the cellulase cellobiohydrolase I (CBH1; [120]). Therefore, *Trichoderma* is considered as an excellent host for the production of heterologous proteins (reviewed in [121,122]). Nyyssönen *et al.* [123] reported a production of 1 mg/L in shake-flasks of Fab antibody fragments by *T. Reesei* Rut-C30. More strikingly, when the Fab antibody fragment chain was fused to the core-linker region of CBH1, a production level of 40 mg/L in shake-flasks and 150 mg/L in bioreactor cultivations was obtained [123,124].

The use of *S. cerevisiae* and *A. awamori* for the large-scale production of antibody fragments and fusion proteins

In our own laboratory at TNO Nutrition and Food Research in Zeist (The Netherlands) and in collaboration with Unilever Research Vlaardingen (The Netherlands) research on antibody fragment production in *S. cerevisiae* and *A. awamori* has been carried out [125,126]. The aim of this project was a detailed comparison of both expression systems, in relation to their possible large-scale production process of antibody fragments and fusion proteins. In the framework of this collaboration also a new *A. awamori* expression system, based on xylose induction was developed [127].

The use of S. cerevisiae *and* A. awamori *for the large-scale production of scFv*

To investigate the feasibility of a large-scale cost-effective process for the extracellular production of (functionalised) scFv fragments initially *S. cerevisiae* was used. However, it was shown that *S. cerevisiae* was a poor host for the production of scFv, since the secretion of scFv was hampered by improper folding of the fragments, because large aggregates were formed in the ER and vacuolar-like organelles. It was hypothesised that the exposure of the hydrophobic surfaces on the V_L and V_H chains of scFv plays an important role in the accumulation of scFv in the cell [128]. Shusta *et al.* [129] reported the increase of scFv production up to 20 mg/L in *S. cerevisiae* by optimising the expression system by overexpression of two ER resident chaperones and reduction of growth temperature. Kauffman *et al.* [130] showed that overexpression of scFv in *S. cerevisiae* resulted in cellular stress, displayed by decreased growth rates and induction of the Unfolded Protein Response (UPR). It was hypothesised that a functional UPR was required to decrease the malfolded scFv in the ER, leading to a recovery from cell stress.

As further improved levels were desired also a fungal expression system was considered [4]. In shake-flask cultures a production level of 10 mg/L was achieved by using *A. awamori* as production host. As secretion of a heterologous protein can be greatly enhanced by fusing it to a "carrier" protein such as glucoamylase (GLA; [117,118]), also this fusion-approach was employed. Analysis of the culture medium of transformants carrying the fusion construct revealed a production of approximately 50 mg/L scFv in the culture medium [4]. Several commercially interesting scFv fragments were investigated for their ability to be produced by *A. awamori* using the GLA-fusion strategy. The results showed that the production levels differed significantly between the different scFv transformants. Interestingly, in some cases increased levels of scFv detected in the culture medium corresponded to an increase of transcription level of the ER chaperone BiPA [131], indicating that the antibody fragments, like in *S. cerevisiae*, may have problems with correct folding and aggregate in the fungal cell.

To increase production levels, successful 10 L and 1,5 × 10^4 L scale fermentations were carried out resulting in 200 mg/L scFv under optimal conditions. However, variable amounts of scFv dimers and other multimers were observed. Recent fermentation experiments performed by Sotiriadis *et al.* [132] showed that the highest scFv level was observed when induction was started in the late exponential phase. An increase of the carbon and nitrogen source concentrations and a decreased of the concentration of the inducer, resulted in increased product yields.

Production of Llama V_{HH} *antibody fragments by* S. cerevisiae *and* A. awamori

Although the production of scFv fragments by *S. cerevisiae* and *A. awamori* was successful, levels up to several g/L were not achieved. Possibly the hydrophobic regions of the scFv, responsible for keeping the variable regions of the heavy and light chains together, could also interact with other molecules in the cell. Aggregation of scFv in *S. cerevisiae* may result in accumulation and subsequent degradation (of a part) of the antibody fragment molecules [128] as frequently observed when expressing heterolo-

gous proteins that exhibit hydrophobic surfaces [133]. Interestingly, antibody fragments devoid of these hydrophobic surfaces could be obtained from camels, dromedaries and llamas (V_{HH}s, see **Llama Heavy-chain antibody fragments (V_{HH}s)** and [13]), providing an option to improve production levels in relevant microorganisms [126].

V_{HH}s could be produced in *E. coli* up to levels of 6 mg/L, were found to be extremely stable, highly soluble and reacted specifically and with high affinity with antigens [52]. V_{HH}s were produced in *S. cerevisiae* at levels over 100 mg/L in shake-flask cultures [134], although considerable amounts of V_{HH}s were detected intracellularly. From a 1,5 × 10^4 L fed-batch fermentation, 1.3 kg of V_{HH}s was obtained, which clearly showed that these fragments could be produced in this host more efficiently than scFv fragments [135]. For a cost-effective large-scale process for the production of V_{HH}s in *S. cerevisiae* further improvement is required. Van der Linden *et al.* [54] showed that production of V_{HH}s by *S. cerevisiae* could be improved by DNA shuffling techniques, in which three homologous V_{HH} genes were randomly fragmentated and reassembled subsequently.

Based on the fact that *A. awamori* performed superior for scFv also the possibility of V_{HH} production by *A. awamori* was investigated. As a model V_{HH}s against the hapten RR6 were chosen [134]. Gene fragments coding for anti-RR6 V_{HH}s were cloned in an expression vector containing the highly inducible endoxylanase promoter. Recent experiments (Joosten *et al.* submitted) showed that functional V_{HH}s could be produced in the culture medium in shake-flask cultures, albeit at relatively low levels. For further optimisation a carrier strategy and controlled fermentations will be carried out.

Production of 'Magic bullets' by A. awamori

A major research interest is the production of fusion proteins or 'Magic bullets', consisting of an antibody fragment (scFv or V_{HH} fragment) fused to an enzyme of interest. In our laboratory research has been carried out with a few examples of scFv fragments coupled to glucose oxidase (GOX). GOX is already for many years an interesting enzyme for coupling to antibodies for killing cells [136]. A scFv, which recognises for example oral *Streptomycetes*, when fused to GOX, which is an antimicrobial enzyme, may kill bacteria by generation of the bactericidal hydrogen peroxide. In activity assays it was shown that the fusion protein produced by *A. awamori* was functional, both in binding to the antigen and GOX activity [4].

In the detergent industry enzymatic bleaching may be a good alternative to the current chemical bleaching used. To make these laundry-cleaning products more effective, the production of Magic bullets by filamentous fungi or yeasts is of interest. An enzyme coupled to an antibody fragment recognising persistent stains from e.g. azo-dyes [134] results in a more directed bleaching process, resulting in lower amounts of required detergent, reduction of harmful effects of the enzyme to the textile and lower environmental burden (see Figure 3).

Currently we are investigating the feasibility of production of V_{HH}-enzyme fusions by *A. awamori*. One of the V_{HH}s used is a model llama V_{HH}, recognising the azo-dye Reactive Red 6 (RR6 [134]). As a bleaching enzyme, the *Arthromyces ramosus* peroxidase (ARP) [137,138] was genetically linked to the V_{HH} fragment. This peroxidase utilises hydrogen peroxide to catalyse the oxidation of a wide range of organic and inorganic compounds, which makes the enzyme suitable for use in bleaching processes [139]. ARP alone could be produced in high amounts by *A. awamori* (800 mg/L; Lokman *et al.* submitted). Preliminary results showed the feasibility of fusion protein production by *A. awamori*, yielding high levels of ARP-V_{HH} fusion protein in controlled fermentation experiments (Joosten *et al.* manuscript in preparation). The fusion protein showed both ARP activity and azo-dye binding activity.

In future experiments V_{HH}s fragments can be replaced by other more relevant antibody fragments, for example those binding tomato or blood spots. Also the peroxidase part of the fusion can be further optimised.

Conclusions and future prospects

Recent developments in the fields of antibody engineering and expression systems have enabled the engineering and production of antibodies and antibody fragments for a wide variety of applications. A lot of examples are already mentioned, but presumably more applications can be envisaged. The development of the 'Magic bullet' approach will even increase the interest in antibodies and their related products, also for applications in human medicine. A recently envisioned application that is of much interest, is the use of antibody fragments in micro-arrays. Antibody arrays can be used for proteomic analysis by comparing the differences in presence of proteins in healthy and diseased cells. For this purpose antibody fragments derived from large phage-antibody libraries can be used as probes to capture proteins on chips in a high-throughput system (reviewed in [140,141]). In this respect, V_{HH}s fragments are of great interest, due to their simple and stable structure.

In this review we evaluated whether the yeast *S. cerevisiae* and the filamentous fungus *A. awamori* are suitable expression systems for the large-scale production of antibody fragments and antibody fusion proteins. Although

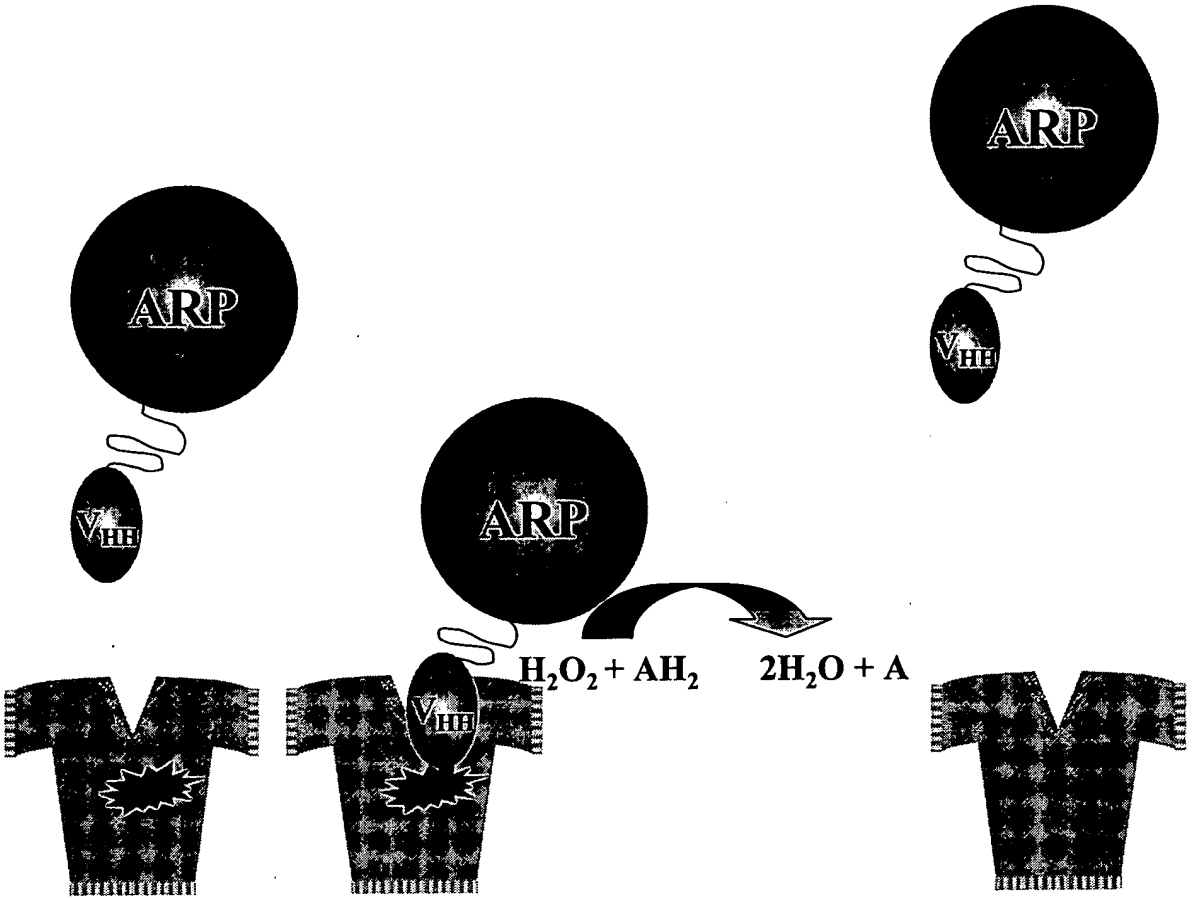

Figure 3
Schematical example of the 'Magic bullet' approach in consumer applications, where an antibody fragment (in this case a llama variable heavy-chain antibody fragment; V_{HH}) recognising a spot on textile, is coupled to an effector molecule (in this case *A. ramosus* peroxidase; ARP). ARP is a peroxidase, which utilises hydrogen peroxide to catalyse the oxidation of a wide range of organic and inorganic compounds, which makes the enzyme suitable for use in bleaching processes [139].

A. awamori is not the best expression system for the production single antibody domains (scFv and V_{HH} fragments), in particular for the production of antibody fusion proteins filamentous fungi offer significant potential. In particular in those cases where specific post-translational modification (*e.g.* N-glycosylation) is required for functional expression of the effector protein (also in relation to pharmaceutical applications). In contrast to *S. cerevisiae*, filamentous fungi do not show extensive hyperglycosylation [142].

Both the scFv-GOX as well as results from ARP-V_{HH} fusion proteins showed that the filamentous fungal system is a promising candidate for the production of antibody fusion proteins. In the future production of other fusion proteins can be investigated in this or other fungal expression systems, allowing a potential breakthrough for antibody technology in producing large amounts of specific recognition units coupled to effector molecules for consumer applications.

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Epitope Mapping

A Practical Approach

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Chapter 1
An introduction to epitope mapping

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Antigens are highly diverse, varying in size as well as the composition of their primary sequence. In the case of proteins, post-translational processing with the addition of moieties, such as carbohydrates during glycosylation, may alter secondary structure. The relative location of epitopes on the surface of an antigen demands ample consideration in relation to the structure when defining and predicting antigenic sites. Although, their identity can only really be found secondarily to the examination of the cellular and humoral products of specific immunity, i.e. lymphocytes and antibodies. Interest in defining epitopes has been borne out, at least in part, from a need for producing non-pathogenic vaccines. At a more technical level, the cloning and sequencing of DNA has allowed the construction of genes for the synthesis of precise antigens in non-human cells. Such advances have obviated the risk of infections, such as hepatitis B and human immunodeficiency virus (HIV) that are potential hazards when preparing antigens from body tissues and fluids.

Antigenic sites for antibody binding are only found on the surface of the molecule and conform to two possible architectural options. An epitope that constitutes part of a linear amino acid sequence on a polypeptide chain is known as a continuous or linear epitope. Where the epitope is formed from two stretches of the primary sequence which are distant from one another, but brought together in the folded molecule's secondary or tertiary structure, then it is known as a conformational epitope.

1 Environmental conditions can influence protein structure

The environment of the whole antigen will affect its molecular folding and thus the nature of epitopes exposed on the molecule's surface. Moreover, the location of the antigen demands consideration since the intracellular conformation differs

from the extracellular counterpart, for a protein passing through the cellular organelles, e.g. endoplasmic reticulum, is required to adopt a loose or open conformation (1). Most naturally occurring proteins are stable, held together in secondary and tertiary structures, i.e. their native conformation, by intramolecular bonds such as hydrogen bonding, hydrophobic, and electrostatic forces. Accordingly these forces may be altered by their environmental conditions, such as pH, temperature, as well as the nature of the solvent in which they are immersed. Treatment with denaturing agents can lead to loss of α-helix or β-pleated structures, converting the protein to a random coil formation and losing its compact structure, for example, breaking disulfide linkage using 2-mercaptoethanol, or the use of urea or guanidinium hydrochloride to alter hydrogen and hydrophobic interactions. When the disulfide and other intramolecule bonds apart from the peptide linkages are broken, the protein is said to be denatured. As a consequence, the molecule may be rendered non-functional if it is less globular, and its physicochemical properties such as solubility and sedimentation rate are altered. The study by Michaelsen *et al.* (1975) is a classic example of how the reduction and alkylation of rabbit immunoglobulins resulted in their diminished capacity to induce cytotoxicity (2). Thus the common use of reducing agents in SDS–polyacrylamide gel electrophoresis (SDS–PAGE) may result in the detection of a different set of epitopes from those exposed on the native protein. For instance, an antibody may not bind to a denatured protein in a Western blot analysis because its structure or shape is different from the native protein. Obviously such factors are of little clinical significance, but such data may be critical for preparing effective diagnostic reagents. Hence it can sometimes be useful to deliberately immunize with the denatured antigen to produce reagents capable of recognizing antigens in SDS–PAGE or in fixed tissue sections.

Ionic strength of the medium affects antibody–antigen interactions *in vitro*, for lower ionic strength solutions will promote antigen–antibody binding. Blood group serologists have exploited this idea for developing shorter incubation times in blood group and cross-matching techniques (3). In this situation, erythrocytes washed and suspended in low ionic strength saline (LISS), are incubated with serum, to ascertain whether atypical blood group antigens are present on the surface of cells that could react with serum antibodies.

1.1 Locating the epitope of the molecule

Any accessible part of a molecule may be considered to be potentially antigenic, some inherently produce a greater response than others. It is a debatable point, but some workers believe that the immune response is largely dependent on the ability of the specific host (4), whereas other suggest that the nature of the antigen is independent of the host being immunized (5). When comparing the immunogenicity of native versus denatured proteins, this is generally a problem for evaluating B cell epitopes rather than for T cell epitopes. It has been well established that immunoglobulins recognize conformation rather than sequence. This somewhat explains why some antibodies will only work in certain assay or analytical systems.

For antibody-binding to an antigen, the specific epitope must be exposed on the molecular surface. There are a number of criteria that might affect position of the epitope, such as:

(a) The mobility of the region of the protein.

(b) Nature of the primary sequence (in the case of protein antigens) where there is the potential to form a loop or turn, e.g. presence of proline residues.

(c) The hydrophilicity.

With reference to molecular mobility of an antigen, X-rays and NMR studies may indicate surface amino acid residues with higher mobility, and areas on the molecule where there are rigid structures, such as α-helices and β-sheets. As a general rule, the hydrophobic stretches of sequence will reside in the interior of the molecule, with the hydrophilic stretches found on the surface. Therefore highly hydrophilic sequences are likely to be on the surface of a molecular fold and thus putative epitopes. When known, the amino acid sequence data is valuable for predicting epitopes, particularly when used in conjunction with hydrophilicity parameters for each residue (6). As far as primary sequences are concerned, the sequence of the amino acids in the peptide chain specify the ultimate three-dimensional (3D) structure. Therefore, from a knowledge of X-ray crystallographic data from other protein sequences that are similar to the protein of interest, its 3D structure may be predicted, and thus so could the exposure of potential epitopes. Moreover, it is a simplistic, but accepted, view that proteins with comparable intermediate or transitional folding during their manufacture and intracellular transport also may have similar native structures (7).

With respect to T cell epitopes it has been generally acknowledged that the T cell receptor recognized amino acid sequence within the peptide groove of the major histocompatibility complex. In this context, it was the nature of the antigen, rather than its shape that was recognized by the T cell arm of the immune system. However, this has since become a subject of some debate. Bach *et al.* (1998) have refuted this evidence in their work on mimic sequence motifs of the pancreatic antigen in insulin-dependent diabetes mellitus, i.e. the 65 kDa glutamic acid decarboxylase (GAD65). They found T cells specific for GAD65 could be stimulated by conformational peptide mimic that had little sequence homology with the defined autoantigenic epitope (8). Their findings have suggested that knowledge of the primary residue contacts with the T cell receptor within the epitope and the MHC class II binding motifs is particularly significant.

The pharmaceutical industry is ever mindful of the need to produce drugs that are easy to produce; having minimum toxicity and that can be rapidly screened for biological activity. This natural concept of mimicry is also being exploited for the design and synthesis of organic compounds that mimic bioactive molecules. Chapter 6 provides protocols that cover the design, preparation, and characterization of peptoids as peptidomimetics.

2 Lessons from an historical perspective

Traditional methods for defining linear epitopes have included fragmentation of proteins either by chemical cleavage, e.g. using cyanogen bromide or by enzymatic digestion. If proteolytic enzymes are to be used, then prior knowledge of the primary sequence is generally required. Where the protein of interest is glycosylated, the removal of post-translational moieties with endoglycosidases may be advisable. Yet post-translational modifications such as oligosaccharide chains of glycoproteins, can form part of an epitope. Alternatively the carbohydrate moiety might even mask an epitope that was present on the native non-glycosylated protein.

2.1 Enzymatic cleavage for epitope mapping

The relative position of an epitope on an intact protein may be detected by limited digestion of the antigen followed by immunoblotting of the protein fragments (9). This may be of clinical relevance when investigating a defect with a protein such as ankyrin—a structural protein with a membrane skeleton where defects may be inherited or induced. In either case, structural changes are liable to result in abnormalities in cellular deformability or fragility (8). Competitive inhibition of epitope binding by synthetic peptides is also used in mapping. But it is expedient for the researcher to be aware that biological activity may be inhibited. Furthermore data may not necessarily allow genuine 'competition' to be distinguished from steric hindrance owing to 'crowding' of the site because of the sheer size of the antibody molecule.

When investigating the nature of putative epitopes on a protein, several enzymatic methods are available, e.g. digestion of the protein into smaller peptide fragments, deglycosylation. The classic work of Rodney Porter (1959) who rationalized the structure of immunoglobulins into their relative areas of activity relied on the hydrolysis of the polypeptide chain at specific positions. By hydrolysis of IgG with papain he produced fragments that bound antigen, i.e. Fab region, and an intact peptide fragment that could be crystallized, i.e. Fc (10).

The purity of both the enzyme preparation as well as the antigen to be digested requires consideration, as does the nature of the protein structure being hydrolysed. Immunoglobulins do not only bind antigen, but also may be considered as antigens. The globular structure of immunoglobulin is a classic example of a molecule that is relatively resistant to enzymatic hydrolysis. The activities of some enzymes are restricted to the hinge region of the molecule where there is some degree of flexibility. Conversely within the Fab region there is a relative absence of movement that can in turn affect hydrolysis. Moreover, the different subclasses of IgG differ with respect to their hinge regions; thus IgG2 and IgG4 that have restricted hinge lengths are resistant to papain hydrolysis (although IgG4 may be hydrolysed in the presence of cysteine). In contrast, IgG1 and IgG3 molecules have flexible hinge regions and are more sensitive to hydrolysis (10).

Carboxypeptidase for C-terminal amino acid sequence analysis has been used

in the investigation of the role of MHC class I molecules in selecting antigenic peptides. These peptides, which are imported into the endoplasmic reticulum for assembly in the MHC class I groove, appear to be protected from cleavage by proteases. One proposal has been that MHC class I molecules are involved in peptide selection by sampling their affinity before protease degradation or efflux into the cytosol (11).

2.2 Protein sequence analysis

To determine the primary sequence of a polypeptide any disulfide bonds must be cleaved, which may be performed using dithiothreitol, or by oxidation with peroxy-formic acid which converts cysteine to cysteic acid (13). Protein that has been purified to homogeneity may then be sequenced from the carboxy (C-) or amino (N-) terminus. C-terminal sequence analysis utilizes the naturally occurring enzyme carboxypeptidase. N-terminal amino acid sequencing is performed by the Edman degradation where the peptide is reacted with phenylisothiocyanate to produce the phenylthiocarbamol derivative. This is subsequently treated with anhydrous hydrochloric acid in an organic solvent to produce a phenylthiohydantoin derivative. The amino acid is separated from the N-terminus of the peptide and identified by chromatography. Partial degradation by chemical cleavage of peptides of proteins has traditionally been used to determine the primary structure. The peptides cleaved may be chromatographically separated, sequenced, and may provide a complete primary sequence (14). To ensure precise data, more than one chemical cleavage protocol may need to be used to produce peptides of differing lengths because they were hydrolysed at specific residue sites. Then the primary sequence may be defined by comparing the overlapping sequences deduced from data obtained from the individual peptide fragments.

Peptides of varying lengths are produced and sequenced: cyanogen bromide selectively cleaves peptides at the carbonyl group of methionine residues, converting the methionine to a C-terminal homoserine lactone unit (15). Epitope mapping using this method was employed by Suphioglu *et al.* (1993) to identify IgE-binding epitopes on the grass pollen allergens of *Pooidae*, *Chloridoideae*, and *Panicoideae* (16). When evaluating the peptide fragments produced from treating the Fc region of IgG with cyanogen bromide, the different allotypes must be considered. For instance G1m(a) has methionine residues at positions 252 and 428, whereas the non-Gm(a) allotype has a methionine at residue 358. Thus cyanogen bromide digestion of these two allotypes produces very different peptide fragments. Other reagents used for the fragmentation of proteins include hydroxyamine that cleaves asparagine–glycine bonds, and 2-nitro-5-thiocyanobenzoate that cleaves peptide chains at the amine side of cysteine residues (17).

Using chemical cleavage produces peptides with different sequences and analysis of the overlapping sequences can produce precise data. Such methods have distinct advantages over enzymatic hydrolysis in that they are not liable to steric hindrance. Moreover, since chemicals are generally smaller molecules than

enzymes, they can penetrate the protein to reach target sites deep within the tertiary structure. Only a limited number of residues can be sequenced at any one cycle owing to 'noise' when evaluating chromatographic peaks. Nevertheless, such information has proved invaluable for extrapolating genetic sequences to synthesize cDNA probes used for identification and isolation of clones from genomic and cDNA libraries.

2.3 Chemical characteristics of sequences

The recognition and binding of an antigen to a lymphocyte receptor is highly dependent on the chemical characteristics of the residues constituting the epitope. Since antigenic epitopes are an integral part of the whole antigen, overall an isolated peptide fragment or synthesized peptide may well have a different conformation from that found when it forms part of the whole antigen. Some amino acids appear to be more immunogenic than others, since they are seen in high frequency as part of antigenic determinants, these include: His, Lys, Ala, Leu, Asp, Arg (in order of decreasing antigenicity). Thus possible antigenic sites may be located by calculating regions of immunogenic potential (18). Knowledge of these parameters would be useful in conjunction with X-ray crystallography data of a named antigen to locate an epitope.

Certain amino acid residues within antigenic epitopes have been shown to be highly significant for binding immunoglobulins. For example, Artandi *et al.* (1992) who analysed specificity of rheumatoid factors, natural autoantibodies whose antigen is the Fc region of IgG, found that the His-435 in the Fc region of the IgG was an important residue for binding (19). A cross-species comparative study of primary sequence data for a named protein could indicate sites of antigenicity, and may improve our understanding of the relative roles of key residues within the protein. From an evolutionary perspective, sites of reactivity may be enhanced, diminished, or totally eliminated.

Optical configuration will also affect immunogenicity; L-forms generally produce a better immune response. One reason is that D-forms of bacterial antigens are not as easily processed by antigen-presenting cells, so are not as effectively presented to T lymphocytes (20).

3 Synthetic peptide technologies for epitope mapping

Geysen *et al.* (1987) investigated possible ways of identifying epitopes on macromolecules like proteins and developed the Pepscan technique, but this requires prior knowledge of amino acid sequence for the protein of interest. Briefly, multiple peptides are synthesized on polystyrene pins; these sequences overlap and span the complete length of the protein. The polystyrene pins are arranged in blocks of 9×12 (96 pins) that may be immersed into the wells of a microtitre plate format. A modified ELISA may be used to screen individual or panels of antibodies for distinguishing linear B cell epitopes (21). The technology and

possible troubleshooting for the analysis of data is clearly covered in Chapter 2. This technique may also be adapted for the delineation of T cell epitopes, both T helper cells (see Chapter 3) and cytotoxic T cell epitope analysis (see Chapter 5). But these authors have also advocated the use of other methodologies including the purification of antigens presented by MHC class I molecules and expression cloning for identifying CTL epitopes following transfection of plasmid DNA into Chinese hamster ovary cells (COS-7 cells) or HeLa cells.

T and B cell epitopes may be overlapping or adjacent within a single sequence, for within a peptide as short as a 10-mer, the respective T and B cell sites may be unique (22). Since B cells can also act as antigen-presenting cells, they are capable of presenting antigen for T helper cell recognition. Accordingly, the cytokine network operates whereby these two immune cell types may augment or dampen the immune response. Thus a single peptide may be able to stimulate both the humoral and cellular arm of immunity, and this has significant implication for possible therapies, e.g. vaccine development. Chapter 4 includes protocols for the identification of combined B and T cell epitopes within a stretch of amino acid sequence using synthetic peptide technologies and complements the chapter on the Pepscan technique (Chapter 2).

Attempts have been made to mimic the shape of conformational epitopes with peptides by building up the structure in iterative stages. First all possible 400 dimeric peptides are synthesized and those showing best binding to antibody are then lengthened at either end with each of the 20 amino acids in turn. Again the best antibody binders are selected and further amino acids added. In this way, with the making of relatively few peptides, mimotopes that mimic conformational structures may sometimes be selected. The technique has had limited success as it has a tendency to select for similar sequences that bind to many different antibodies. The technique with random phage sequences, described in Chapter 9, where full-length peptides are used at the first selection, has proved more useful to generate unique structures for individual antibodies.

4 Chemical modification of antigens

Chemical modifying reagents with the ability to penetrate reaction sites have been strategically employed to investigate potential active interaction sites. It is well documented that changes to side chains on macromolecules, e.g. proteins, immunoglobulins, can alter antigenicity and/or biological function. For instance, Boc-Cys(Npys)-OH blocks the thiol groups of cysteine residues and can render T cell epitopes inactive (23).

Immunoglobulins are excellent candidates for investigating antigenicity since they have a number of active sites, e.g. binding sites for the Fc receptor, complement. The IgG molecule is the antigen for naturally occurring antibodies, called rheumatoid factors (RFs) that are detected in a number of different connective tissue disorders, e.g. rheumatoid arthritis, systemic lupus erythematosus (24, 25). To examine the possible binding sites for RFs, IgG has been chemically modified

by carbamylation: this involves the treatment of IgG with 2 M potassium cyanate and the subsequent modification of α and ε amino groups, particularly affecting lysine residues. Conflicting evidence has been obtained regarding changes in antigenicity of carbamylated IgG; whilst some workers have not found changes in polyclonal antisera reactivity (26), others have suggested that changes in reactivity of the Fc region could be detected (27). These discrepancies are not surprising, since changes in antigenicity would be dependent on the extent of chemical modification and whether the carbamylation had provoked changes in the epitope *per se* and/or the molecular conformation. This is not simply a chemical artefact that happens *in vitro*, for the addition of isocyanic acid (a degradation product of urea), i.e. carbamylation of proteins, happens *in vivo*, e.g. erythrocyte membrane proteins of uraemic patients (28).

Nitration is another example of how the use of one technique is very much antigen-dependent. For example, nitrated human IgG failed to react with rheumatoid factors, whereas nitrated rabbit IgG maintained reactivity. Such discrepancies were attributed to differences in amino acid sequences between rabbit and human IgG that led to changes in conformation post-treatment. This in turn affected the antigenic site where there are tyrosine residues that are directly involved in the rheumatoid factor-binding of human IgG (26). Tetranitromethane modifies tyrosine residues but there are some limitations:

(a) The extent of treatment is governed by the concentration of the reagent used in the reaction mixture.

(b) Possible phylogenic variation in the structure of proteins.

5 Site-directed mutagenesis as a tool for epitope mapping

With knowledge of the amino acid sequence data for a protein of interest, the precise residues that form the epitope involved in immune recognition may be determined. Where a protein exhibits evolutionary variation or where there is the possibility of mutants, this divergence will inevitably govern its 'foreignness' to the host, and thus the subsequent immune response. When the protein being studied is conserved across species, often the antigen produces a limited immune response. The most probable rationale is that antigen is not perceived as 'dangerous', instead it is tolerated like so many other 'self'-antigens.

From a purely academic perspective, the study of natural variants has demonstrated some success in mapping epitopes on protein antigens, but the technique has its limitations:

(a) The number of different proteins available for study is restricted to ones where there are multiple species isolates available, moreover, are these proteins of any real interest?

(b) Researchers are limited to studying the few amino acid variations that occur naturally rather than numerous strategic changes along the primary sequence.

(c) Single amino acid substitutions rarely occur in isolation. Even where a protein has been conserved across species there is likely to be a number of sequence differences and this may result in variation within protein conformation, and thus the nature of the epitope exposed on the antigen surface. Therefore when analysing the between-species variation of a named antigen and its binding to a specific antibody, any discrepancy may not be the contribution of a single residue on the antigen surface, but also the effects of other amino acid substitutions.

Site-directed mutagenesis is a powerful tool that solves all of these problems for it allows any single amino acid within a protein sequence to be substituted with another. When an individual residue is changed, the effect of this change on antibody binding can be assessed by a variety of techniques (29). Unlike the case with natural variants or mutants, with site-directed mutagenesis, the role of individual amino acids within the immune complex formed may be evaluated. For example, an amino acid may be substituted to produce a variation in side chain, electrostatic charge (smaller size residue), or one that produces a definite change in conformation, e.g. proline. This allows an in-depth analysis of the contribution of specific amino acids to the energy of complex formation.

To determine the residues involved in complex formation, the technique of alanine-scanning mutagenesis may be employed. In this instance, single point mutants of a protein are systematically generated to assess the molecular interactions at several protein–protein interfaces (30). Alanine is an amino acid with a very simple structure, i.e. only has a single methyl group as the side chain (which compares to the carbon in the side chains of all amino acids except glycine and proline). So alanine substitution has the advantage of effectively removing from the antigen–antibody interface the energetic contributions of all side chain atoms beyond the C position, including intramolecular forces, e.g. side chain hydrogen bonds. Accordingly, this technique affords the researcher the ability to evaluate the contribution of individual amino acids at the antigen–antibody interface. Perdue (Chapter 10) has given an excellent overview and examples of the concept of site-directed mutagenesis, providing the researcher with a general body of knowledge from which to obtain further information. The methods given are straightforward and troubleshooting points clear for those wishing to use these techniques.

6 Hybridoma technology and epitope analysis

Chapter 7 provides detailed protocols for production of monoclonal antibody probes. Monoclonal antibodies (mAbs) are themselves powerful reagents in diagnostic applications and as tools for the investigation of macromolecules and cells (31, 32), and permit standardization and production of an unlimited supply of a reagent. This in turn may be used to set up a technique where the antibody used is of a high titre and the protocol is highly reproducible. Conversely, polyclonal

reagents are limited in supply and generally of low titre, and there is likely to be between-batch variation.

6.1 Protein footprinting in epitope analysis

Hybridoma technology for the production of monoclonal antibodies has the added advantage that being epitope-specific, these antibodies in turn may be used in the study of protein structure. The technique of protein footprinting relies on the fact that the epitope is protected from proteolytic or chemical cleavage when bound as an antibody–antigen complex, and the protected epitope may then be eluted (33). Albeit data on the primary structure are not needed, ultimately knowledge of the tertiary conformation is useful if the location of the epitope on the whole molecule is required. Obviously with a larger antigen, the physicochemical interaction that stabilizes the epitope and its interaction with the antibody binding site are also not available. Much information on the interface of antigen–antibody interactions has been derived from X-ray crystallographic studies, and again, lysozyme as the model antigen in association with the monoclonal antibody has been extensively studied (34).

7 Generating monoclonal antibody

Nelson (Chapter 7) has commented on the reactivity of monoclonal antibodies to desired target antigens, and looks at how this may be applied to the development of secure assay systems and reliable functional studies. The more traditional techniques (enzymatic or chemical degradation studies) for localizing target epitope may be used in conjunction with analysing mAb reactivity in assay systems, e.g. enzyme-linked immunosorbent assay, haemagglutination, and slot-blotting. Likewise *in situ* localization of cell surface or intracellular antigenic determinants may be achieved by immunocytochemical techniques. Again it is essential to point out that mAbs, like polyclonal antisera, only bind if the epitope conformation is maintained. Conversely, if the assay system or the technique in which it is utilized, e.g. antigen denaturation in SDS–PAGE prior to Western blot analysis, modifies the epitope, then a positive antigen–antibody complex may not be detected. Evidently, other methodologies, e.g. for isolating cell membrane components and protein purification steps are required, and it is suggested that these protocols are obtained from other sources (35–37). Ultimately, epitope localization can be further refined by epitope mapping studies which specifically highlight salient residues (38). Nelson has provided information on where techniques have been applied as well as useful information on company addresses and financial costing for researchers considering developing monoclonal reagents.

8 Phage display libraries

Phage display libraries bring together two methodologies, linking the genetic and amino acid sequence of a peptide. The technique involves the cloning of

oligonucleotide sequences that encode peptides via their ligation into a vector DNA whilst preserving the reading frame of the phage fusion protein. The filamentous bacteriophage then displays the gene product, i.e. peptide encoded by the oligonucleotide, as a fused protein on the bacteriophage coat protein. Accordingly, transfection of bacteriophage into a known recipient bacterial strain means distinct peptides that mimic native epitopes, i.e. 'mimotopes' are displayed. These random phage display libraries may be screened using an antibody, then the nature of the peptide elucidated by DNA sequencing to confirm the amino acid sequence of specific epitope. Such libraries have the distinct advantage that positive bacteriophage particles (with respect to antibody binding) may be amplified in bacterial culture. In addition, there is the opportunity for further analysis of individual phage clones such as phage ELISAs. Chapter 9 shows how a peptide library made up of nine random amino acids could be used to delineate the epitopes recognized by anti-β-lactoglobulin antibodies. Although the preliminary studies using phage display libraries evaluated the epitopes of monoclonal antibodies, recently, Williams *et al.* have shown how the same technique may be applied to looking for immunodominant epitopes of polyclonal antisera, and they were also able to compare their technique directly with data obtained using a Pepscan assay (synthetic peptide library on plastic pins) that they used to screen the same antiserum. Moreover, they have suggested possible uses including the generation of human antisera for use as pharmaceutical agents. Certainly biological libraries such as those with phage display have the advantage of:

(a) Being able to be propagated almost *ad infinitum*.

(b) Numerous selection cycles may be performed to purify and enhance a rare ligand.

(c) Since sequence analysis of the isolated bacteriophage particles is straightforward, the selection for binding activity also means selecting for the encoding gene.

(d) Synthetic libraries do not have the 'biological selection' bias towards a particular amino acid sequence that may occur *in vivo*. Moreover the peptides used are not individually synthesized, therefore their conformation is not affected by the rest of the bacteriopnage coat protein.

9 Carbohydrates and their significance when epitope mapping

It was Landsteiner whose interest in blood group serology led to the investigation of cell surface carbohydrate antigens (ABO blood group system) on erythrocytes, long before the structure of immunoglobulin molecules had been determined. Glycosylation of physiologically active molecules is often essential for their functionality. Oligosaccharide moieties fulfil a number of biological roles, including the protection of the core protein from proteolytic attack, induction and maintenance of the active site configuration, and may or may not decrease immuno-

genicity. Deglycosylation may lead to loss of antigenicity if the sugars form part of the epitope, or alternatively if the charge and/or conformation of the active epitope is modified following their removal. For instance, aglycosylated IgG has a reduced capacity for complement- and FcγR-mediated processes. Changes in IgG glycosylation have been associated with some autoimmune diseases, e.g. rheumatoid arthritis, where the terminal galactose is missing in up to 60% of IgG molecules—the so-called $\%G_{(0)}$ which can be determined by lectin affinity studies (39). This methodology relies on the binding of galactose molecules to *Ricinus communis* agglutinin, and *N*-acetyl glucosamine to *Bandeiraea simplicifolia* II. The ratio of binding to these two lectins may be used to calculate the $G_{(0)}$ in IgG. Glycosylation changes in IgG have been implicated as contributing to immune complex formation. Westwood *et al.* (1994) proposed that putative epitopes that are partially masked by the oligosaccharides in normal IgG and exposed agalactosyl IgG epitopes are therefore available as targets for rheumatoid factor-binding (40).

Some physiological molecules exist naturally in glycosylated and non-glycosylated forms. Epitope mapping to assess any possible minor differences in configuration may be profitable when investigating receptor–ligand interactions and drug design, as well as (where appropriate) evaluating physiological function. Prolactin, a polypeptide hormone produced and secreted by the anterior pituitary gland and endometrium, is one such example. The glycosylated form is the major form produced by the endometrium during the menstrual cycle. The release of prolactin by the pituitary is stimulated by thyrotrophin-releasing factor, but inhibited by the drug bromocriptene, yet both have no effect on the release by the endometrium (41). Although the role of this protein is not fully understood, it is possible that minor changes in molecular structure that delineates the different isoforms may augment or diminish biological activity (42).

Broadly there are two methods available for the removal of oligosaccharides from proteins: chemical or enzymatic hydrolysis. Endoglycosidases such as neuraminidase (that hydrolyses sialic acid) may be utilized, but they are notoriously inefficient with limited activity, depending on the source of the enzyme (43). Chemical deglycosylation involves using trifluormethane sulfonic acid. This is a highly aggressive method that again is not necessarily 100% effective at removing the carbohydrate, and can also affect the integrity of the protein core (44).

In the assessment and characterization of carbohydrate moieties, the researcher may need knowledge of:

(a) The oligosaccharide sequence.

(b) The nature of the binding of carbohydrate to the protein (N-linked or O-linked).

(c) The intramolecular interactions between the protein and the carbohydrate that can in turn affect conformation and charge.

Chapter 8 gives information and protocols on how carbohydrate libraries (collections of chemically modified carbohydrates) may be employed in the epitope mapping of carbohydrate binding proteins, as well as their use as probes. In

addition they have also given examples of how data may be interpreted with respect to molecular interactions.

10 Approaches to epitope mapping

The best approach to defining antigenic epitopes depends mainly on the resources available but some factors merit consideration before beginning analysis.

10.1 Polyclonal or monoclonal antibody?

In most circumstances the end-result of experimentation is to understand how whole animals or individuals are responding to antigens. In order to do this polyclonal systems require investigation. The results obtained from epitope mapping with antisera can, however, be horrendously complicated to analyse. Even supposedly non-immune sera can give multiple positive spikes when analysed in Pepscan assays. Monoclonal antibodies are much cleaner to work with but have the disadvantage that they may be non-representative of the antibody population as a whole.

10.2 Whole antigen available?

A great deal can be learned about the likely position of epitopes, in their native conformation, by carrying out initial studies to try to localize and chemically characterize principal sites. Enzymic or chemical fragmentation to identify key areas; denaturation to reveal linear versus conformational epitopes; deglycosylation to pin-point carbohydrate-dependent sites; specific amino acid modification to highlight key residues; crystallography to enable prediction from likely surface structures.

10.3 Amino acid sequence known?

Algorithmic predictions of likely epitopes can cut down the number of peptides that need to be synthesized; the full sequence then allows the power of Pepscan to be applied.

10.4 Nucleotide sequence available?

The ease and comparative cheapness of phage display makes this a favoured approach especially as the epitope is displayed in the context of a whole protein. Site-directed mutagenesis then allows the key residues in the identified epitope to be delineated.

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CHAPTER 135

VON WILLEBRAND DISEASE

DAVID GINSBURG

von Willebrand factor (vWF) is a central component of hemostasis, serving both as a carrier for factor VIII and as an adhesive link between platelets and the injured blood vessel wall. Abnormalities in vWF function result in von Willebrand disease (vWD), the most common inherited bleeding disorder in humans. The overall prevalence of vWD has been estimated to be as high as 1 percent of the general population, although the prevalence of clinically significant disease is probably closer to 1:1000. vWD is associated with either quantitative deficiency (type 1 and type 3) or qualitative abnormalities of vWF (type 2). The uncommon type 3 variant is the most severe form of vWD and is characterized by very low or undetectable levels of vWF, a severe bleeding diathesis, and a generally autosomal recessive pattern of inheritance. Type 1 vWD, the most common variant, is characterized by vWF that is normal in structure and function but decreased in quantity (in the range of 20 to 50 percent of normal). In type 2 vWD, the vWF is abnormal in structure and/or function. Type 2A vWD is associated with selective loss of the largest and most functionally active vWF multimers. Type 2A is further subdivided into group 1, due to mutations that interfere with biosynthesis and secretion, and group 2, in which the mutant vWF exhibits an increased sensitivity to proteolysis in plasma. Type 2N vWD is characterized by mutations within the factor VIII binding domain of vWF, leading to disproportionately decreased factor VIII and a disorder resembling mild hemophilia A, but with autosomal recessive inheritance. Type 2B vWD is due to mutations clustered within the vWF A1 domain, in a segment critical for binding to the platelet glycoprotein Ib (GPIb) receptor. These mutations produce a "gain of function" resulting in spontaneous vWF binding to platelets and clearance of the resulting platelet complexes, leading to thrombocytopenia and loss of the most active (large) vWF multimers. Type 1 vWD can often be effectively managed by treatment with DDAVP, which produces a two- to threefold increase in plasma vWF level. Response to DDAVP is generally poor in type 3 and most of the type 2 vWD variants. These disorders often require treatment with factor replacement in the form of plasma or selected factor VIII concentrates containing large quantities of intact vWF multimers.

DEFINITION AND HISTORY

In 1926, Eric von Willebrand described a bleeding disorder in 24 of 66 members of a family from the Åland Islands.[1] Both sexes were afflicted, and the bleeding time was prolonged despite normal platelet counts and normal clot retraction. von Willebrand distinguished this condition from the other hemostatic diseases known at the time and recognized its genetic basis, calling the disorder "hereditary pseudohemophilia," but incorrectly characterizing the inheritance as X-linked dominant. von Willebrand's confusion about the inheritance pattern was probably due, at least in part, to the greater recognition of bleeding symptoms in women because of the hemostatic stresses of menstruation and parturition. The proband in the original family, Hjördis, was 5 years old at the time of von Willebrand's initial evaluation and ultimately died at age 13 during her fourth menstrual cycle. Four of Hjördis' sisters died between the ages of 2 and 4, and deaths in the family were also noted during childbirth.

An apparently similar disorder was independently reported in the United States by Minot and others in 1928. The original family in the Åland Islands was reexamined by von Willebrand and Jürgens in 1933, leading to the conclusion that the defect in this disorder was due to an impairment of platelet function. It was not until 1953 that Alexander and Goldstein demonstrated reduced levels of coagulation factor VIII in vWD patients, along with prolonged bleeding time. This observation was confirmed by others, including studies of the original von Willebrand pedigree by Nilsson and coworkers. In the late 1950s, the latter group demonstrated that a fraction of plasma referred to as "I-O" could correct the factor VIII deficiency and normalize the bleeding time, indicating that the defect in vWD was due to the deficiency of a plasma factor rather than an intrinsic platelet abnormality. Infusion of fraction I-O promptly increased the factor VIII level in a hemophilic patient, while in vWD the factor VIII level rose gradually, peaking at 5 to 8 h. Fraction I-O prepared from a hemophilia A patient was also shown to correct the defect in vWD, demonstrating that these disorders were due to deficiencies of distinct plasma factors (reviewed in refs. 2 and 3).

It was not until 1971 that Zimmerman, Ratnoff, and Powell prepared the first antibodies against what was thought to be a highly purified form of factor VIII.[4] This factor VIII–related antigen was found to be normal in hemophilia A patients but decreased in vWD. This puzzle was finally resolved with the demonstration that vWF and factor VIII are closely associated, with over 98 percent of the mass of the complex composed of vWF (see below). Thus, antibodies raised against this complex predominantly recognize vWF. The first direct assay of vWF function was based on the observation that the antibiotic ristocetin induced thrombocytopenia and the demonstration by Howard and Firkin[5] that ristocetin-induced platelet aggregation was absent in some vWD patients. Weiss and coworkers[6] used this observation to develop a quantitative assay for vWF function that remains a mainstay of laboratory evaluation for vWD to this day. In 1973, several groups succeeded in dissociating vWF from factor VIII procoagulant activity.[7,8]

Final proof that vWF and factor VIII are independent proteins encoded by distinct genes came with the cDNA cloning of the two molecules in 1984 and 1985.[9–14] These discoveries also marked the beginning of the molecular genetic era for the study of vWF and factor VIII, leading to the identification of gene mutations in many patients with hemophilia and vWD as well as considerable insight into the structure and function of these related proteins.

Table 135-1 summarizes the current nomenclature and terminology for factor VIII and vWF. vWD is a heterogenous disorder with over 20 distinct variants described. The previous complex and confusing classification has recently been consolidated and simplified,[15,16] as summarized in Table 135-2. Type 3 vWD is associated with very low or undetectable levels of vWF and severe bleeding. Type 1 vWD is characterized by concordant reductions in factor VIII activity, vWF antigen, and ristocetin cofactor activity, generally to the range of 20 to 50 percent of normal, in association with normal vWF multimer

Acronyms and abbreviations that appear in this chapter include: aPPT, activated partial thromboplastin time; DDAVP, 1-desamino-8-D-arginine vasopressin, or desmopressin; ER, endoplasmic reticulum; GPIb, glycoprotein Ib; HHT, hereditary hemorrhagic telangiectasia; PCR, polymerase chain reaction; RIPA, ristocetin-induced platelet aggregation; vWD, von Willebrand disease; vWF, von Willebrand factor.

TABLE 135-1 VON WILLEBRAND FACTOR AND FACTOR VIII TERMINOLOGY

Factor VIII
Antihemophilic factor, the protein that is reduced in plasma of patients with classic hemophilia A and vWD and is measured in standard coagulation assays
Factor VIII activity (factor VIII:C)
The coagulant property of the factor VIII protein (this term is sometimes used interchangeably with factor VIII)
Factor VIII antigen (VIII:Ag)
The antigenic determinant(s) on factor VIII measured by immunoassays, which may employ polyclonal or monoclonal antibodies
von Willebrand factor (vWF)
The large multimeric glycoprotein that is necessary for normal platelet adhesion, a normal bleeding time, and stabilizing factor VIII
von Willebrand factor antigen (vWF:Ag)
The antigenic determinant(s) on vWF measured by immunoassays, which may employ polyclonal or monoclonal antibodies; *inaccurate designations of historical interest only* include factor VIII-related antigen (VIIIR:Ag), factor VIII antigen, AHF antigen, and AHF-like antigen
Ristocetin cofactor activity
The property of vWF that supports ristocetin-induced agglutination of washed or fixed normal platelets

structure. Type 2 vWD is heterogeneous and further divided into four subtypes (2A, 2B, 2N, and 2M). Type 2A vWD is characterized by a disproportionately low level of ristocetin cofactor activity relative to vWF antigen and absence of large and intermediate-sized multimers. Type 2B vWD is also associated with reduced high-molecular-weight vWF multimers, but as the result of an abnormal vWF molecule with increased affinity for platelet GPIb. Abnormalities in vWF that result in decreased factor VIII binding to vWF have also been described (type 2N) and present as mild to moderate factor VIII deficiency. Many other subtypes have been reported, including platelet-type (pseudo-) vWD, which is actually an intrinsic platelet disorder due to mutations in GPIb (see Chap. 119). Finally, acquired forms of vWD also occur, generally due to autoantibody formation.

ETIOLOGY AND PATHOGENESIS

vWF is synthesized exclusively in endothelial cells and megakaryocytes and performs two major functions in hemostasis. First, vWF serves as the initial critical bridge between circulating platelets and the injured blood vessel wall, accounting for the apparent defect in platelet function and prolonged bleeding time observed in vWD patients. The vWF monomer is assembled into higher-order multimers, a structure required for optimal adhesive function. Second, vWF serves as the carrier in plasma for factor VIII, ensuring its stability and localizing it to the initial platelet plug for participation in thrombin generation and fibrin clot formation (see Chap. 112). This tight, noncovalent interaction between vWF and factor VIII accounts for the copurification of these two molecules and the resulting initial confusion as to the origin of hemophilia and vWD. Factor VIII is encoded by the factor VIII gene on the X chromosome (see Chaps. 112 and 123), while vWF is encoded by a distinct gene on human chromosome 12.

THE VON WILLEBRAND FACTOR GENE AND cDNA

The vWF cDNA was initially cloned from endothelial cells[11–14] and the corresponding gene mapped to the short arm of chromosome 12 (12p13.3).[11] The vWF mRNA is approximately 9.0 kb in length, encoding a primary translation product of 2813 amino acid residues with an estimated M_r of 310,000. Comparison of the primary peptide sequence obtained from plasma vWF[17] with the vWF cDNA sequence established the pre-propolypeptide nature of vWF.[18] Pre-propolypeptide vWF is composed of a 22–amino acid signal peptide, a 741–amino acid precursor polypeptide (propeptide) termed *vWF antigen II*, and the mature subunit.[11,18–21] Cleavage of the 741–amino acid propeptide from the amino terminus produces the mature vWF subunit of 2050 amino acids (Fig. 135-1).

Analysis of the vWF sequence identifies four distinct types of repeated domains: three A domains, three B domains, two C domains, and four D domains.[19,22] The first pair of D domains is tandemly arranged in the vWF propeptide, followed by a partial and full D domain at the N terminus of the mature subunit. The final complete D domain is separated by a segment of more than 600 amino acids containing the triplicated A domains. The repeated domain structure of vWF suggests that the gene may have evolved via a complex series of partial duplications, although exon structure is not highly conserved between homologous domains.

Comparison of the vWF amino acid sequence to other proteins identifies a superfamily of related proteins that all share sequence similarity with the vWF A domains.[23] The common theme among these potentially evolutionarily related genes is a role in extracellular matrix or adhesive function. Consistent with this notion, vWF functional domains for binding to the platelet receptor GPIb and specific ligands within the extracellular matrix have been localized to the vWF

TABLE 135-2 CLASSIFICATION OF VON WILLEBRAND DISEASE

Type	Inheritance	Frequency	Factor VIII Activity	vWF Antigen	Ristocetin Cofactor Activity	RIPA	Plasma vWF Multimer Structure	Previous Nomenclature
Type 1	Autosomal dominant	1–30:1000; most common vWD variant (>70% of vWD)	Decreased	Decreased	Decreased	Decreased or normal	Normal	Type I
Type 3	Autosomal recessive (or codominant)	$1–5:10^6$	Markedly decreased	Very low or absent	Very low or absent	Absent	Usually absent	Type III
Type 2A	Usually autosomal dominant	≈10–15% of clinically significant vWD	Decreased to normal	Usually low	Markedly decreased	Decreased	Largest and intermediate multimers absent	Type IIA, IB, I "platelet discordant," IIC–H
Type 2B	Autosomal dominant	Uncommon variant (<5% of clinical vWD)	Decreased to normal	Usually low	Decreased to normal	Increased to low concentrations of ristocetin	Largest multimers absent	Type IIB
Type 2M	Usually autosomal dominant	Rare (case reports)	Variably decreased	Variably decreased	Decreased	Variably decreased	Normal	Type B, IC, ID, Vicenza
Type 2N	Autosomal recessive	Uncommon; heterozygotes may be prevalent in some populations	Decreased	Normal	Normal	Normal	Normal	vWD Normandy
Platelet-type (pseudo-)	Autosomal dominant	Rare	Decreased to normal	Decreased to normal	Decreased	Increased to low concentrations of ristocetin	Largest multimers absent	

A repeats. A potential relationship between the vWF C domains and portions of thrombospondin and procollagen has also been proposed.[24]

The vWF gene spans approximately 180 kb and is divided into 52 exons.[25] Exons range in size from 40 bases to 1.5 kb (exon 28). The latter exon is unusually large, encoding the entire A1 and A2 domains and containing nearly all of the known type 2A and type 2B vWD mutations. The concentration of these defects within one exon has facilitated the identification of human mutations responsible for these vWD variants (see Molecular Genetics of von Willebrand Disease, below). A partial, nonfunctional duplication of the vWF gene, termed a pseudogene, has been localized to human chromosome 22.[26] The pseudogene duplicates the middle portion of the vWF gene, from exons 23 to 34, and includes the intervening sequences. The pseudogene is approximately 97 percent identical in sequence to the authentic vWF gene, indicating that it is of fairly recent evolutionary origin.[27]

vWF is synthesized exclusively in megakaryocytes and endothelial cells and, as a result, has frequently been used as a specific histochemical marker to identify cells of endothelial cell origin. Although generally assumed to mark all endothelial cells, vWF is expressed at widely varying levels among endothelial cells, depending on the size and location of the associated blood vessel.[28,29] A recent careful survey in the mouse identified wide differences in the level of vWF mRNA, with 5 to 50 times higher concentrations in the lung and brain, particularly in small vessels, than in comparable vessels in the liver and kidney. In general, the higher levels of vWF mRNA and antigen were found in the endothelial cells of large vessels rather than in microvessels and in venous rather than arterial endothelial cells.[29]

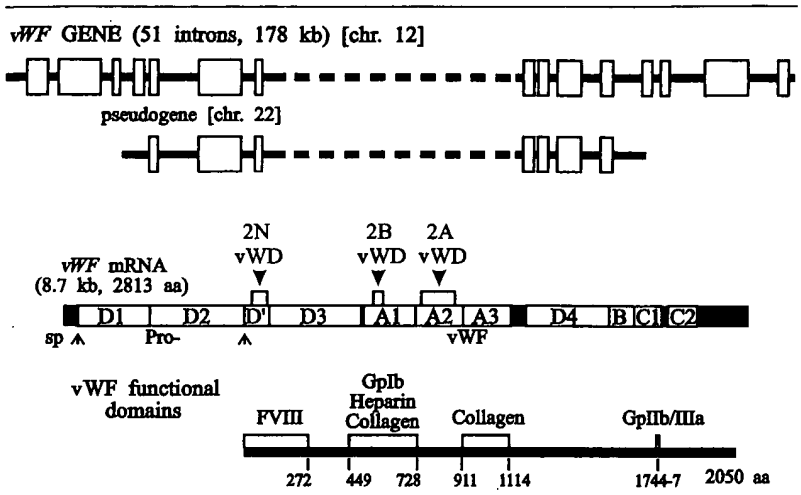

FIGURE 135-1 Schematic diagram of the human vWF gene, mRNA, and protein. The vWF gene and pseudogene are depicted at the top, with boxes representing exons and the solid black line introns. The vWF mRNA encoding the full prepro-vWF subunit is depicted in the middle as the bar and lettered boxes. The locations of signal peptide (sp) and propeptide (Pro) cleavage sites are indicated by arrowheads, and the lettered boxes denote regions of internally repeated sequence. The approximate localizations for known vWF functional domains within the mature vWF subunit are indicated at the bottom. Numbers underneath the domains refer to amino acid residues within the mature vWF subunit. The clusters of mutations responsible for type 2A, type 2B, and type 2N vWD are indicated. (aa, amino acids; chr, chromosome.) (Adapted from D Ginsburg and EJW Bowie[222] with permission.)

Specific DNA sequences within or near the proximal promoter of the vWF gene appear to be required for endothelium-specific gene expression,[30] although it is likely that additional important regulatory elements exist outside of this region, perhaps at a great distance. A portion of the human vWF promoter from −487 to +246 has been shown to target vWF expression to blood vessels of the yolk sac and a subset of endothelial cells in the adult brain of the mouse.[31] This heterogeneity in expression level among different endothelial cell subsets has only recently been appreciated.[32]

VON WILLEBRAND FACTOR BIOSYNTHESIS

The processing steps involved in the biosynthesis of vWF are similar in megakaryocytes[33] and endothelial cells[34–36] (reviewed in ref. 37). vWF is first synthesized as a large, precursor monomer polypeptide, depicted schematically in Fig. 135-1. vWF is unusually rich in cysteine, which accounts for 8.3 percent of its amino acid content. All cysteines in the mature vWF molecule are involved in disulfide bonds.[38] Pro-vWF monomers are assembled into dimers through disulfide bonds at both C termini, and only dimers are exported from the endoplasmic reticulum (ER).[38–40]

Glycosylation begins in the ER, with 12 potential *N*-linked glycosylation sites present on the mature subunit and 3 on the propeptide. Extensive additional posttranslational modification of vWF occurs in the Golgi apparatus, including the addition of multiple *O*-linked carbohydrate structures, sulfation, and multimerization through the formation of disulfide bonds at the N termini of adjacent dimers. vWF is the only protein known to undergo extensive disulfide bond formation at this late stage, and this unique process appears to be catalyzed by a novel disulfide isomerase activity present within the vWF propeptide.[41] Mutations at either of two specific cysteines within the propeptide that are thought to be critical for disulfide isomerase activity, or a shift in the spacing between them, results in loss of multimer formation.[41] The multimerization process appears to require the slightly acidic environment of the distal Golgi.[42] The vWF propeptide self-associates and may also serve to align vWF subunits for multimer assembly.[43] However, the propeptide facilitates multimer assembly even when coexpressed as a separate molecule from the mature vWF monomer.[44,45]

Propeptide cleavage occurs late in vWF synthesis or just prior to secretion. Cleavage occurs adjacent to two basic amino acids, Lys-Arg at positions −2 and −1. An Arg at position −4 is also required for recognition by the intracellular protease responsible for propeptide cleavage.[46] Multimerization and propeptide cleavage are not linked to each other. The multimers secreted by cultured endothelial cells contain both pro-vWF and mature subunits,[36,47] and recombinant vWF with a point mutation inhibiting propeptide cleavage is still assembled into normal multimer structures.[48] Although propeptide cleavage appears to occur primarily intracellularly, cleavage may also occur after secretion.

vWF is secreted from endothelial cells via both constitutive and regulated pathways.[37] vWF is stored in tubular structures within the α granule of platelets and within the Weibel-Palade body in endothelial cells.[49–52] Weibel-Palade bodies are derived from the Golgi apparatus and are found in most endothelial cells, though the number varies considerably. Although a number of other hemostatic proteins are also stored in the platelet α granule, the Weibel-Palade body appears to be relatively specific for vWF and its propeptide.[53,54] The transmembrane glycoprotein P-selectin is also found in the membranes of both the α granule and the Weibel-Palade body.[55] The only other known component of the Weibel-Palade body is CD63, a lysosomal protein also found on activated platelets.[56,57]

Regulated secretion of vWF from its storage site in the Weibel-Palade body is triggered by a number of secretagogues, including thrombin,[58] fibrin,[59] histamine,[60] and the C5b-9 complement complex.[61]

While the vasopressin analog desmopressin acetate (DDAVP) causes marked release of vWF in vivo, it has no direct effect on endothelial cells in culture,[62] suggesting that its effects are controlled by a secondary mediator. Constitutive secretion of vWF occurs evenly at the apical and basolateral surface, while regulated secretion from the Weibel-Palade body is highly polarized in the basolateral direction.[63] While constitutively secreted multimers are of relatively small size, the multimers stored within the Weibel-Palade body are the largest, most biologically potent form.[54,64] The vWF stored in platelet α-granules is also enriched for large multimers.[65] The N-terminal D domains appear to be required for vWF storage, with deletion of any of the individual domains resulting in constituent secretion.[66,67] It also appears that cleavage of the vWF prosequence is required for efficient formation of storage granules.[68]

The concentration of vWF in plasma is approximately 10 μg/ml, with approximately 15 percent of circulating vWF localized to the platelet compartment.[69] Bone marrow transplants between normal and vWD pigs demonstrate that platelet vWF is derived entirely from synthesis within the marrow and does not contribute to the normal plasma vWF pool.[70–72] These studies also demonstrate that both the plasma and the platelet vWF pools are required for full hemostasis, although the plasma pool appears to be more critical.

Plasma vWF appears to be further processed through cleavage by a specific protease in the circulation, resulting in reduction in the size of the largest multimers.[73,74] The major proteolytic cleavage site has been mapped to the peptide bond between Tyr842 and Met843 in the vWF A2 domain,[75] and recombinant vWF missing the A2 domain is resistant to proteolysis.[76] vWF carrying a subgroup of type 2A vWD mutations exhibits increased susceptibility to cleavage by this protease,[77] and this is the proposed mechanism for the selective loss of large vWF multimers in this group of patients (see Molecular Genetics of von Willebrand Disease, below). Recently, this specific vWF processing protease activity has been proposed to play a central role in the pathophysiology of chronic relapsing thrombotic thrombocytopenic purpura (see Chap. 117).[78–80]

THE FUNCTION OF VON WILLEBRAND FACTOR

vWF is a large multivalent adhesive protein that plays an important role in platelet attachment to subendothelial surfaces, platelet spreading, and platelet-platelet interactions, that is, aggregation at sites of vessel injury. vWF also stabilizes factor VIII. The interaction of vWF and factor VIII is important for the protection of factor VIII from inactivation or degradation. Factor VIII bound to vWF may localize to cells and/or sites where it can more readily participate in the promotion of blood coagulation and/or thrombus formation.

vWF is required for the adhesion of platelets to the subendothelium, particularly at moderate to high shear force. vWF performs this bridging function by binding to two platelet receptors, GPIb and GPIIb/IIIa, as well as to specific ligands within the exposed subendothelium at sites of vascular injury (reviewed in refs. 81–83). Binding of vWF to its platelet receptors generally does not occur in the circulation under normal conditions. However, the interaction of vWF with its ligands in the vessel wall, combined with high shear stress conditions, facilitates vWF binding to platelet GPIb and subsequent platelet adhesion and activation. Activation of platelets leads to the exposure of the GPIIb/IIIa complex, an integrin receptor that can bind to fibrinogen and other ligands, including vWF, to form the platelet-platelet bridges required for thrombus growth. Platelet adhesion to vWF immobilized at a site of injury appears to be a two-step process, with the initial tethering of the rapidly moving platelet dependent on the vWF/GPIb interaction and subsequent firm adhesion occurring through GPIIb/IIIa after platelet activation.[84,85]

von WILLEBRAND FACTOR BINDING TO THE VESSEL WALL

vWF binds to several different types of collagens, including types I through VI. Two distinct binding domains for the fibrillar collagens, types I and III, have been localized to specific segments within the vWF A1 and A3 repeats (see Fig. 135-1),[86,87] and a potential third domain has been identified in the propeptide.[88] Studies of recombinant vWF suggest that the A3 collagen-binding domain may be the most important.[89,90] The physiologic relevance of vWF interactions with fibrillar collagens has been questioned, since vWF still binds to extracellular matrix depleted of these molecules by treatment with collagenase.[91] vWF has also been shown to bind to the nonfibrillar collagen type VI, which is resistant to collagenase[92] and colocalizes with vWF in the subendothelium.[93] Type VI collagen supports the binding of vWF under high shear through cooperative interactions between binding domains within the vWF A1 and A3 repeat.[94] Although vWF binding has also been demonstrated in a number of other potential components of the subendothelium, including glycosaminoglycans[95,96] and sulfatides,[97] the biologic significance of these interactions remains to be demonstrated.

von WILLEBRAND FACTOR BINDING TO PLATELETS

vWF interacts with a receptor complex on the surface of platelets composed of the disulfide-linked GPIbα and GPIbβ chains noncovalently associated with GPIX and GPV. The binding site for vWF is within a 293–amino acid segment at the N terminus of GPIb and requires sulfation of several key tyrosine residues for optimal binding.[98] The GPIb binding domain within vWF has been mapped to the A1 segment, within the disulfide loop formed between the cysteine residues at 509 and 695 (see Fig. 135-1).[99,100] Ristocetin binds to both vWF and platelets, but the mechanism by which it enhances the vWF/GPIb interaction is still poorly understood.[101,102] The snake venom botrocetin appears to induce GPIb binding through a different alteration in the vWF A1 domain and is also used to study this interaction.[103] Scanning mutagenesis studies of recombinant vWF have characterized a number of amino acid residues within the vWF A1 domain that are critical for binding to GPIb and for interaction with botrocetin.[104] Several mutations were also identified that increase platelet binding, an effect similar to that of mutations associated with type 2B vWD (see Molecular Genetics of von Willebrand Disease, below). These natural and synthetic mutations cluster in a small area on the surface of the vWF A1 domain structure, as revealed by x-ray crystallographic studies.[105] The structure of the A1 domain closely resembles that of other previously studied A domains, including the vWF A3 domain.[106,107]

The structure of the vWF A1 domain and the mutations that cause enhanced GPIb binding, together with the observations that these mutations enhance binding to similar extents and are not additive when inserted into the same molecule, suggest an allosteric "on-off" model for vWF function.[83,108] In this model, the vWF GPIb binding domain is in an "off" configuration when at rest in the circulation. Binding to collagen (or another ligand in the vessel wall) or interaction with soluble modulators such as ristocetin or botrocetin induces a switch to the "on" conformation, resulting in platelet binding. In an alternative model, it is the immobilization of vWF on a surface, together with high shear force, that facilitates the multivalent interaction of vWF with the platelet surface, rather than a specific conformational switch within the vWF A1 domain.[82,109]

The Arg-Gly-Asp-Ser (RGDS) sequence at amino acids 1744–1747 of the mature vWF subunit serves as the binding site within vWF for GPIIb/IIIa. The latter complex, also known as α_{IIb}/β_3, is a member of the integrin family of cell surface receptors. GPIIb/IIIa undergoes a conformational change to a high-affinity ligand-binding state following platelet activation and, in addition to vWF, can bind

a number of other adhesive proteins, including fibrinogen. Although vWF is present in blood at much lower concentrations than is fibrinogen, evidence suggests that vWF may be a critical ligand under flow conditions.[84,85] An RGD sequence is also present in the vWF propeptide (vWF antigen II), although its functional significance is unknown.

THE INTERACTION OF von WILLEBRAND FACTOR WITH FACTOR VIII

The noncovalent interaction between factor VIII and vWF is required for the stability of factor VIII in the circulation, as is evident from the factor VIII levels of less than 10 percent that are observed in most severe vWD patients. Although each vWF subunit appears to carry a binding site for factor VIII, the stoichiometry for the vWF/FVIII complex found in normal plasma is approximately 1 to 2 factor VIII molecules per 100 vWF monomers.[110] Factor VIII bound to vWF is also protected from proteolytic degradation by activated protein C (reviewed in refs. 83 and 111).

The factor VIII binding domain within vWF has been localized to the first 272 N-terminal amino acids of the mature subunit,[112] with antibody studies suggesting a particularly critical role for amino acids 78–96.[113,114] The mutations identified in patients with type 2N vWD, in which vWF binding to factor VIII is specifically affected (see Molecular Genetics of von Willebrand Disease, below), are all clustered in this region, including the most common type 2N mutation, at Arg91.[115] It is noteworthy that the same amino acid substitution at Arg89 is a common polymorphism that does not affect factor VIII binding.[116] The corresponding binding site for vWF on factor VIII includes an acidic region at the N terminus of the light chain (residues 1669–1689)[117] and requires sulfation of Tyr1680 for optimal binding.[118] Thrombin cleavage after Arg1689 activates and releases factor VIII from vWF. Thus, vWF may serve to efficiently deliver factor VIII to the sites of clot formation, where it can complex with factor IXa on the platelet surface.

MOLECULAR GENETICS OF VON WILLEBRAND DISEASE

vWD is an extremely heterogenous and complex disorder, with over 20 distinct subtypes reported (reviewed in refs. 83, 119, and 120). A large number of mutations within the vWF gene have now been identified (Fig. 135-2). A partial list is maintained by a consortium of vWD investigators and can be accessed through the internet at http://mmg2.im.med.umich.edu/vWF.[121,122] These findings form the basis for the simplified classification of vWD outlined in Table 135-2[15,16] and used throughout this chapter. Types 1 and 3 vWD are defined as pure quantitative deficiencies of vWF that are either partial (type 1) or complete (type 3). Type 2 vWD is characterized by qualitative abnormalities of vWF structure and/or function. The quantity of vWF found in type 2 vWD may be normal, but it is usually mildly to moderately decreased (see Table 135-2).

TYPE 3 von WILLEBRAND DISEASE

Patients with type 3 vWD have very low or undetectable levels of plasma and platelet vWF antigen and ristocetin cofactor activity and generally present early in life with severe bleeding.[123] Factor VIII coagulant activity is markedly reduced but usually detectable at levels of 3 to 10 percent of normal. Type 3 vWD appears to be inherited as an autosomal recessive trait in most families, but parents of affected individuals may have mildly reduced vWF levels and are occasionally given the diagnosis of mild type 1 vWD.

Southern blot analysis has identified gross gene deletion as the molecular mechanism for type 3 vWD in only a small subset of families[26,124–126]; however, large deletions may confer an increased risk for the development of alloantibodies against vWF.[26,126] A similar correlation has been reported for hemophilia B (see Chap. 123). Comparative analysis of vWF genomic DNA and platelet vWF mRNA has identified nondeletion defects resulting in complete loss of vWF mRNA expression as a molecular mechanism in some patients with type 3 vWD.[127,128] A number of nonsense and frameshift mutations that would be predicted to result in loss of vWF protein expression or in expression of a markedly truncated or disrupted protein have been identified in some type 3 vWD families (see Fig. 135-2).[119,121,129,130] A frameshift mutation in exon 18 appears to be a particularly common cause of type 3 vWD in the Swedish population and has been shown to be the defect responsible for vWD in the original Åland Island pedigree.[131,132] This mutation results in a stable mRNA encoding a truncated protein that is rapidly degraded in the cell.[133] This mutation also appears to be common among type 3 vWD patients in Germany[134] but not in the United States.[133]

TYPE 1 von WILLEBRAND DISEASE

Type 1 is the most common form, accounting for approximately 70 percent of vWD patients. Type 1 vWD is generally autosomal dominant in inheritance and is associated with coordinate reductions in factor VIII, ristocetin cofactor activity, and vWF antigen with maintenance of the full complement of multimers (Fig. 135-3). Subgroups within type 1 vWD have been proposed based on the relative levels of vWF present in the plasma and platelet pools.[135–138]

Type 1 vWD is generally assumed to simply represent the heterozygous form of type 3 vWD. However, the majority of heterozygous carriers of vWF gene deletions, as well as carriers of vWF mRNA expression defects,[26,124,125,127,128] are asymptomatic and have normal vWF laboratory values, consistent with an autosomal recessive pattern of inheritance for type 3 vWD. Nonetheless, in some families with nonsense or frameshift mutations, heterozygotes with apparent type 1 vWD have been identified, indicating that some or all type 1 vWD may be due to such defects within the vWF gene (see Fig. 135-2). Mutations that give rise to defective vWF subunits that interfere in a dominant negative way with the normal allele may be particularly likely to cause symptomatic vWD in the heterozygote.[130] Mutations have been identified at several cysteine residues in the vWF D3 domain of patients with moderately severe type 1 vWD. vWF carrying one of these mutations was shown to be retained in the ER and appeared to exert a dominant negative effect on the normal vWF allele.[139]

To date, all mutation studies and genetic linkage analysis of type 1 vWD have been consistent with defects within the vWF gene (reviewed in ref. 130). However, the possibility that a subset of type 1 vWD is due to defects in genes outside of vWF (locus heterogeneity) must still be considered. Given the complex biosynthesis and processing of vWF, defects at a number of other loci could be expected to result in quantitative vWF abnormalities. However, no such example has yet been reported. It is interesting to note that a mouse model for type 1 vWD associated with an up to twentyfold reduction in plasma vWF is due to an unusual mutation in a glycosyltransferase gene, leading to aberrant posttranslation processing of vWF and accelerated clearance from plasma.[140] A similar mechanism may explain the modifying effect of the ABO blood group glycosyltransferases on plasma vWF level.[141]

TYPE 2A von WILLEBRAND DISEASE

Type 2A is the most common qualitative variant of vWD and is generally associated with autosomal dominant inheritance and selective loss of the large and intermediate vWF multimers from plasma (see Fig. 135-3). A 176-kDa proteolytic fragment present in normal individuals is markedly increased in quantity in many type 2A vWD patients. This fragment is due to proteolytic cleavage of the peptide bond between Tyr842 and Met843.[75,142] Based on this observation,

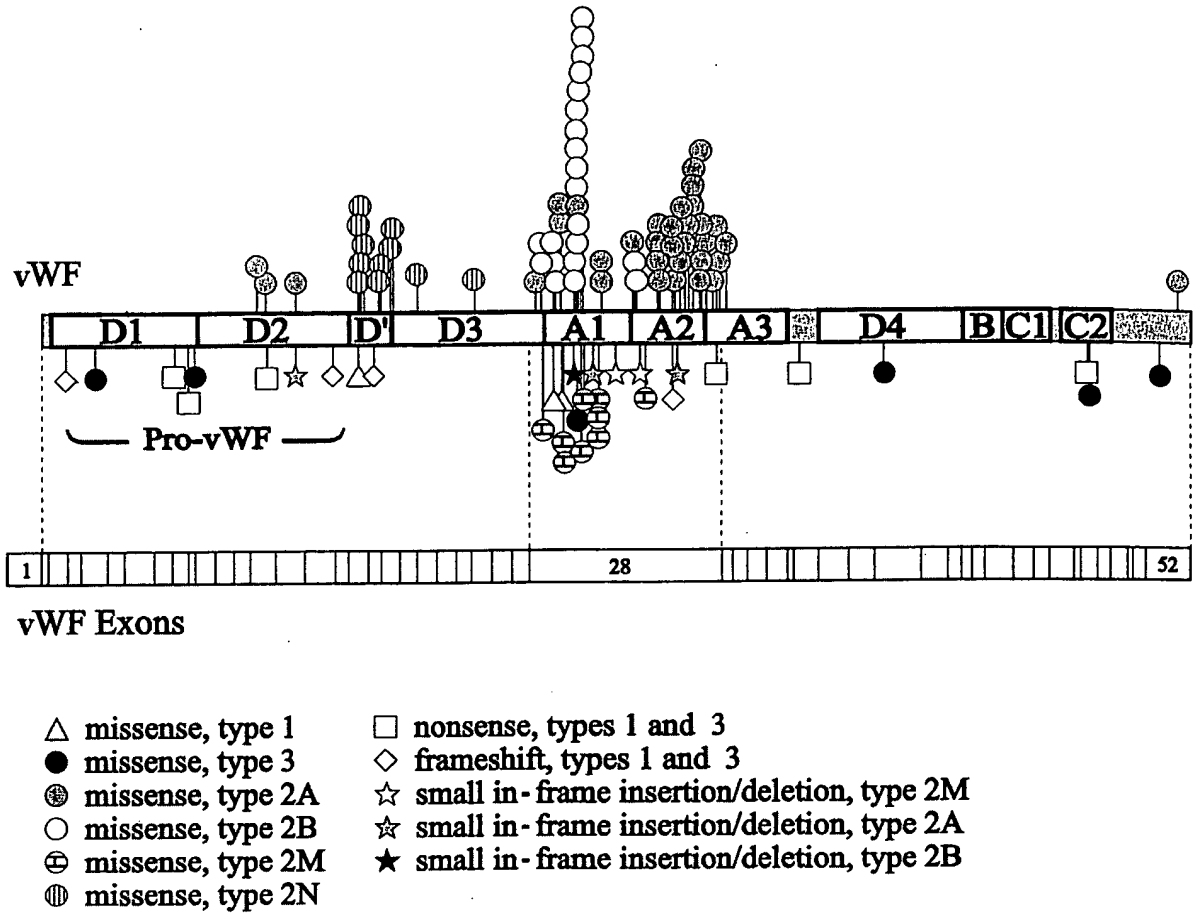

FIGURE 135-2 vWD mutations. The location of all point mutations and small, in-frame insertions and/or deletions associated with vWD, as reported to the vWD database (*http://mmg2.im.med.umich.edu/vWF*), are depicted within the vWF coding sequence. Shown below are the relative positions of all 52 vWF gene exons. (Adapted from WC Nichols and D Ginsburg[119] with permission.)

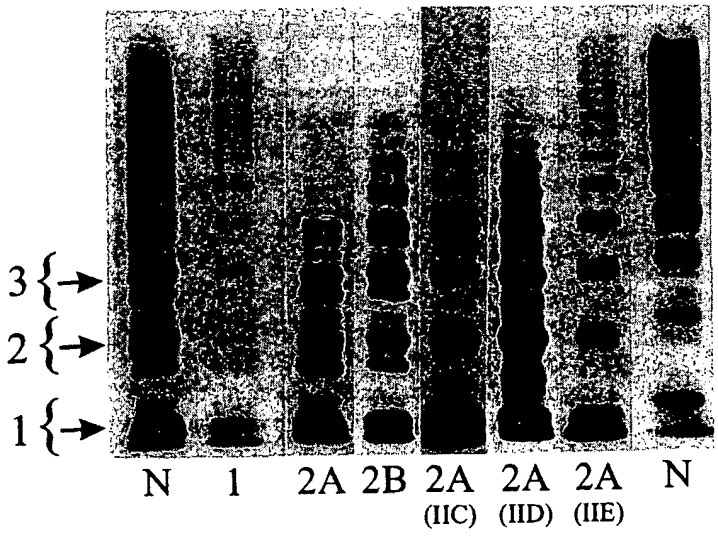

FIGURE 135-3 Agarose gel electrophoresis of plasma vWF. vWF multimers from plasma of patients with various subtypes of vWD are shown. The brackets to the left encompass three individual multimer subunits, including the main band and its associate satellite bands. N indicates normal control lanes. Lanes 5 through 7 are rare variants of type 2A vWD. The former designations for these variants are indicated in parentheses below the lanes (IIC through E). (Adapted from SD Berkowitz et al[223] with permission.)

initial DNA sequence analysis in patients centered on vWF exon 28, in the region encoding this segment of the vWF protein, leading to the identification of the first point mutations responsible for vWD.[143] Since that time, a large number of mutations have been identified, accounting for the majority of type 2A vWD patients.[119] Most of these mutations are clustered within a 134–amino acid segment of the vWF A2 domain (between Gly742 and Glu875; see Fig. 135-3), and the most common, Arg834Trp, appears to account for about one-third of type 2A vWD patients.[119,121]

Expression of recombinant vWF containing type 2A vWD mutations has identified two distinct molecular mechanisms for the loss of large vWF multimers characteristic of this disorder.[144] In the first subset, classified as group 1, the type 2A vWD mutation results in a defect in intracellular transport, with retention of mutant vWF in the ER. In the second subset, or group 2, mutant vWF is normally processed and secreted in vitro, and thus loss of multimers in vivo presumably occurs due to increased susceptibility to proteolysis in plasma.[75,144–147] As noted above, the protease that appears to be responsible for this cleavage has been identified.[73,74,77]

The multimer structure of platelet vWF correlates well with this subclassification. Group 1 patients show loss of large vWF multimers within platelets due to defective synthesis, while group 2 patients have normal vWF multimers within the protected environment of the α granule.[144] These observations confirm the earlier subclassification of type 2A vWD based on platelet multimers.[135] Subclassification into group 1 or 2 might be expected to predict response to desmopressin therapy, although this remains to be demonstrated.

In addition to the major class of type 2A vWD described above, a number of rare variants previously classified as types IIC–H, type IB, and "platelet discordant" are now included in the new, more general type 2A category. Most of these rare variants were distinguished on the basis of subtle differences in the multimer pattern (see Fig. 135-3; reviewed in ref. 120). The IIC variant is usually inherited as an autosomal recessive trait and is associated with loss of large multimers and a prominent dimer band. Several mutations have been identified in the vWF propeptide of these patients,[148,149] presumably interfering with multimer assembly. A mutation at the C terminus of vWF, interfering with dimer formation, was recently described in a patient with the IID variant.[150] Most of the other reported variants of type 2A vWD are quite rare, often limited to single case reports.

TYPE 2B von WILLEBRAND DISEASE

Type 2B vWD is usually inherited as an autosomal dominant disorder and is characterized by thrombocytopenia and loss of large vWF multimers. The plasma vWF in type 2B vWD binds to normal platelets in the presence of lower concentrations of ristocetin than does normal vWF and often binds spontaneously. Accelerated clearance of the resulting complexes between platelets and the large, most adhesive forms of vWF accounts for the thrombocytopenia and the characteristic multimer pattern (see Fig. 135-3).

The peculiar functional abnormality characteristic of type 2B vWD suggested a molecular defect within the GPIb binding domain of vWF. For this reason, initial DNA sequence analysis focused on the corresponding portion of vWF exon 28.[151,152] Nearly all of these mutations are located within the vWF A1 domain, at one surface of the recently described crystallographic structure.[105] The four most common mutations are clustered within a 35–amino acid stretch between Arg543 and Arg578 (see Fig. 135-2); together, these account for more than 80 percent of type 2B vWD patients.[121] Functional analysis of mutant recombinant vWF[108,153–156] confirms that these single–amino acid substitutions are sufficient to account for increased GPIb binding and the resulting characteristic type 2B vWD phenotype.

Three families have been described that exhibit enhanced vWF binding to GPIb but a normal distribution of vWF multimers. These variants, previously referred to as type I New York, type I Malmö, and type I Sydney, are now all designated as type 2B vWD. Type I New York and type I Malmö have now been shown to be due to the same mutation, Pro503Leu. This mutation is located within the cluster of type 2B mutations in the vWF A1 domain and results in a similar increase in platelet GPIb binding.[157]

TYPE 2N von WILLEBRAND DISEASE

As described in Chap. 123, hemophilia A results from defects in the factor VIII gene and is inherited in an X-linked recessive manner. Rare families have been reported in which the inheritance of hemophilia appears to be autosomal, based on the occurrence of affected females or direct transmission from an affected father.[158,159] Several cases of an apparent autosomal recessive decrease in factor VIII have been shown to be due to decreased binding of factor VIII by vWF.[160–162] This disorder has also been referred to as vWD Normandy, after the province of origin of the first patient. DNA sequence analysis has identified a total of 11 mutations associated with this disorder, all located at the vWF N terminus (see Fig. 135-2).[115,120] One of these mutations, Arg91Gln, appears to be particularly common and may contribute to variability in the severity of type 1 vWD in some cases.[163]

TYPE 2M von WILLEBRAND DISEASE

This category is reserved for rare vWD variants in which a defect in vWF platelet-dependent function leads to significant bleeding but vWF multimer structure is not affected (although some have subtle multimer abnormalities). Most of these variants were previously classified as type I. The variant previously referred to as type B is associated with absent ristocetin cofactor activity but normal platelet binding with other agonists. This variant has been shown to be due to a mutation in the A1 domain (Gly561Ser).[164] Mutations have also been identified in a number of other families with normal vWF multimers and disproportionately decreased ristocetin cofactor activity.[120,165] Several families have been described with a vWD variant (vWD Vicenza) characterized by larger than normal vWF multimers.[166] Although the mutation responsible for this disorder has not been identified, genetic linkage analysis indicates that the defect lies within the vWF gene.[167]

CLINICAL FEATURES

INHERITANCE

Type 1, the most common form of vWD, is generally transmitted as an autosomal dominant disorder and accounts for approximately 70 percent of clinically significant vWD. However, disease expressivity is variable, and penetrance is incomplete.[130] Laboratory values and clinical symptoms can vary considerably, even within the same individual, and establishing a definite diagnosis of vWD is often difficult. In two large families with type 1 vWD, only 65 percent of individuals with both an affected parent and an affected descendent had significant clinical symptoms.[168] For comparison, 23 percent of the unrelated spouses of the patients, who presumably did not have a bleeding disorder, were judged to have a positive bleeding history.

A number of factors are known to modify vWF levels, including ABO blood group, Lewis antigen, estrogens, thyroid hormone, age, and stress.[169,170] ABO blood group is the best characterized of these factors. Mean vWF antigen levels for type O individuals are approximately 75 percent and for type AB individuals 123 percent when compared to a pool of normal donor plasmas. Thus, it may be difficult to differentiate between a low-normal value and mild type 1 vWD in blood group O individuals.[169] The variable expressivity and incomplete penetrance of type 1 vWD has complicated the determination of accurate incidence figures for vWD. The prevalence of type 1 vWD has been estimated to be as high as 1 percent and as low as 3 to 4 per 100,000.[171,172]

In general, the type 2 variants are more uniformly penetrant. Type 2A and type 2B vWD account for the vast majority of patients with qualitative vWF abnormalities. No accurate incidence figures are available for these subtypes, but the type 2 variants are generally felt to comprise 20 to 30 percent of all vWD diagnoses. The type 2 variants are generally autosomal dominant in inheritance, although rare cases of apparent recessive inheritance have been reported.

Estimates of prevalence for severe (type 3) vWD range from 0.5 to 5.3 per 1,000,000.[173–175] Although this variant is frequently defined as autosomal recessive in inheritance, this is not a consistent finding. As described above, one or both parents of a severe vWD patient are frequently clinically asymptomatic and often have entirely normal laboratory test results, but many families have also been reported in which one or both parents appear to be affected with classic type 1 vWD. Thus, in some families, severe vWD may represent the homozygous form of type 1 vWD. In this model, the apparent recessive inheritance in a subset of families could simply be the result of the incomplete penetrance of type 1 vWD. Alternatively, there may be a fundamental difference in the molecular mechanisms responsible for type 1 and type 3 vWD.[130]

CLINICAL SYMPTOMS

Mucocutaneous bleeding is the most common symptom in patients with type 1 vWD.[168] It is important to note that over 20 percent

of normal individuals may give a positive bleeding history.[176] This observation, together with the limited sensitivity and specificity of the currently available laboratory tests (see below), makes the diagnosis of mild vWD quite difficult and probably contributes to the wide range of prevalence figures for type 1 vWD currently in the literature.

Epistaxis occurs in approximately 60 percent of type 1 vWD patients, 40 percent have easy bruising and hematomas, 35 percent have menorrhagia, and 35 percent have gingival bleeding. Gastrointestinal bleeding occurs in approximately 10 percent of patients.[177] An apparent association between hereditary hemorrhagic telangiectasia (HHT) and vWD had been reported in several families. Genetic defects causing HHT have been localized to chromosomes 3, 9, and 12 (see Chap. 123), and thus most cases are unlikely to be linked to the vWF gene on chromosome 12. However, since inheriting vWD is likely to increase the severity of bleeding from HHT, the diagnosis is more likely to be made in patients inheriting both defects.[178] Mucocutaneous bleeding is common after trauma, with about 50 percent of patients reporting bleeding after dental extraction, about 35 percent after trauma or wounds, 25 percent postpartum, and 20 percent postoperatively. Spontaneous atraumatic hemarthroses occur almost exclusively in patients with type 3 vWD. Hemarthroses in patients with moderate disease are extremely rare and are generally only encountered after major trauma.

Patients with type 3 vWD can suffer from severe clinical bleeding and experience hemarthroses and muscle hematomas, as in severe hemophilia A (see Chap. 123). The bleeding time is very prolonged. After infusion of vWF-containing plasma fractions, some of these patients develop anti-vWF antibodies that neutralize vWF. Development of antibodies has been correlated with the presence of gene deletions.[26,126]

The bleeding symptoms can be quite variable among patients within the same family and even in the same patient over time. An individual may experience postpartum bleeding with one pregnancy but not with others, and clinical symptoms in mildly to moderately affected type 1 individuals often ameliorate by the second or third decade of life. Aside from an infrequent type 3 patient, death from bleeding rarely occurs in vWD.

Thrombocytopenia is a common feature of type 2B vWD and is not seen in any other form of vWD. Most patients only experience thrombocytopenia at times of increased vWF production or secretion, such as during physical effort, in pregnancy, in newborn infants, postoperatively, or if an infection develops. The platelet count rarely drops sufficiently to contribute to clinical bleeding.[179,180] Infants with type 2B vWD may present with neonatal thrombocytopenia, which could be confused with neonatal sepsis or congenital thrombocytopenia.

Patients who are homozygous or compound heterozygous for type 2N vWD generally have normal levels of vWF antigen and ristocetin cofactor activity and normal vWF platelet adhesive function. However, factor VIII levels are moderately decreased, resulting in a mild to moderate hemophilia-like phenotype.[115] However, in contrast to patients with classic hemophilia A (factor VIII deficiency), these patients do not respond to infusion of purified factor VIII and should be treated with vWF-containing concentrates. Heterozygotes for this disorder may have mildly decreased factor VIII levels but are generally asymptomatic. Although type 2N vWD appears to be considerably less common than classic hemophilia A, it should be considered in the differential diagnosis of factor VIII deficiency, particularly if any features suggest an autosomal pattern of inheritance. Although the factor VIII level rarely drops below 5 percent, at least one type 2N vWD mutation has been associated with factor VIII levels as low as 1 percent, when coinherited with a type 3 vWD allele.[181] The latter observation suggests that a diagnosis of type 2N vWD should also be considered in patients with marked reductions of factor VIII.

LABORATORY FEATURES

In the initial laboratory evaluation of patients suspected by history of having vWD, the following tests are routinely performed: assay of factor VIII activity, vWF antigen (vWF:Ag), and ristocetin cofactor activity. In a large epidemiologic study, the ristocetin cofactor assay was found to be more sensitive than the vWF:Ag for the diagnosis of type 1 vWD.[182] Other tests that are commonly used include the bleeding time, ristocetin-induced platelet aggregation (RIPA), and vWF multimer analysis. As noted above, results of these tests can all be normal in some patients with type 1 vWD. In addition, the wide range of normal and the considerable overlap with the levels observed in type 1 vWD make borderline levels difficult to interpret. A variety of concurrent diseases and drugs may modify the results of individual tests, including aspirin or other nonsteroidal anti-inflammatory drugs, which often prolong the bleeding time. Many conditions, such as pregnancy, time of the menstrual cycle, hypo- or hyperthyroidism, uremia, recent exercise, liver disease, infection, diabetes, estrogen therapy, or myeloproliferative syndromes, affect the factor VIII activity, vWF:Ag, and ristocetin cofactor activity levels. These values can be regarded as acute-phase reactants, and many minor illnesses can increase their levels to normal. Even controlling for many of these factors, the coefficients of variation of repeated vWF:Ag and ristocetin cofactor assays in a single person are quite large.[183] For this reason, repeated measurements are usually necessary, and the diagnosis of vWD or its exclusion should not be based on a single set of laboratory values unless they are well below or well above the limits of normal.

BLEEDING TIME

The bleeding time has long been used as a standard screening test for vWD and other abnormalities of platelet function.[184] However, results can vary considerably with the experience of the operator and a variety of other factors, and its value as a screening test has been questioned. There is now a general consensus that the bleeding time should not be used for routine patient screening in the preoperative setting.[185–187] While the bleeding time should also probably not be used as a routine screening test for vWD, it may still be of value in selected patients when taken together with the clinical history and the results of other laboratory tests. It may also be useful as a means of monitoring therapy in some settings.

FACTOR VIII

Factor VIII levels in vWD patients are generally coordinately decreased along with plasma vWF. Levels in type 3 vWD generally range from 3 to 10 percent. In contrast, the levels in type 1 and the type 2 vWD variants (other than 2N) are variable and usually only mildly or moderately decreased. The factor VIII level in type 2N vWD is more severely decreased, but rarely to less than 5 percent. The activated partial thromboplastin time (aPTT) can be prolonged in vWD, although only as a reflection of the reduced factor VIII level.

VON WILLEBRAND FACTOR ANTIGEN

Plasma vWF:Ag is usually quantitated by electroimmunoassay, radioimmunoassay, or an ELISA technique. In type 1 vWD, the vWF:Ag assay usually parallels the ristocetin cofactor activity, but it has lower specificity and sensitivity than the ristocetin cofactor assay.

In patients with type 2A vWD, the vWF:Ag is usually low but can be normal.[183]

RISTOCETIN COFACTOR ACTIVITY

The standard measure of vWF activity quantitates the ability of plasma vWF to agglutinate platelets in the presence of ristocetin,[188] also referred to as the ristocetin cofactor assay. Normal platelets washed free of plasma vWF are used either as fresh platelets or after formaldehyde fixation. This assay appears to be the most sensitive and specific single test for the detection of vWD.[182] While it is generally decreased coordinately with vWF:Ag and factor VIII in type 1 vWD patients, ristocetin cofactor activity is usually disproportionately decreased in the type 2A variants, due to the greater dependence of the latter assay on the larger vWF multimers.

A specific assay of FVIII binding to vWF has been developed and is used to confirm the diagnosis of type 2N vWD.[189] Although this assay is widely used in European hemostasis laboratories, its availability in the United States is currently limited to a few specialized reference laboratories.

A number of other assays for vWF activity have been proposed, including measurement of platelet agglutination induced by botrocetin and other snake venom proteins,[190] assays based on collagen binding,[191] and a new functional assay that measures platelet binding under high shear.[192] While the latter device shows some promise, none of these assays is currently available in the routine clinical laboratory.

RISTOCETIN-INDUCED PLATELET AGGLUTINATION (RIPA)

The addition of ristocetin to normal platelet-rich plasma causes platelet clumping. This activity is generally reduced in most vWD patients. Hyperresponsiveness to ristocetin-induced platelet agglutination results either from a type 2B vWD mutation or an intrinsic defect in the platelet (platelet-type or pseudo-vWD). In these disorders, patient platelet-rich plasma agglutinates spontaneously or at ristocetin concentrations of only 0.2 to 0.7 mg/ml. At these concentrations, normal platelet-rich plasma does not agglutinate. Type 2B and platelet-type vWD can be distinguished by RIPA experiments performed with separated patient platelets or plasma mixed with the corresponding component from a normal individual.

MULTIMER ANALYSIS

Analysis of plasma vWF multimers is critical for the proper diagnosis and subclassification of vWD (see Fig. 135-3). This is generally accomplished by agarose gel electrophoresis of plasma vWF to separate vWF multimers on the basis of molecular size, with the largest multimers migrating more slowly than the intermediate or smaller multimers. The multimers may be visualized by autoradiography after incubation with ^{125}I-monospecific anti-human vWF antibody or by nonradioactive immunologic techniques. The normal multimeric distribution is an orderly ladder of major protein bands of increasing molecular weight, going from the smallest to the largest vWF multimers (see Fig. 135-3). Each normal multimer has a fine structure consisting of one major component and two to four satellite bands.[193] Type 2B and most of the type 2A variants were initially distinguished from each other on the basis of subtle variations in the satellite band pattern.

DIFFERENTIAL DIAGNOSIS

PRENATAL DIAGNOSIS

Given the mild clinical phenotype of most patients with the common variants of vWD, prenatal diagnosis for the purpose of deciding on terminating the pregnancy is rarely performed. However, type 3 vWD patients often have a profound bleeding disorder, similar to or more severe than classic hemophilia, and so some families may request prenatal diagnosis. In those cases of vWD in which the precise mutation is known, DNA diagnosis can be performed rapidly and accurately by PCR from amniotic fluid or chorionic villus biopsies.[194] In those cases where the mutation is unknown, diagnosis can still be attempted by genetic linkage analysis using the large panel of known polymorphisms within the vWF gene.[122] One of these polymorphisms, a TCTA tetranucleotide repeat of variable length in intron 40, is particularly useful, with over 100 known polymorphic alleles. Several cases of successful prenatal diagnosis have been reported.[194–196] Although all cases of vWD analyzed to date appear to be linked to the vWF gene, the possibility of locus heterogeneity (i.e., a similar phenotype due to a mutation in a gene other than vWF) should be considered.[130]

DNA DIAGNOSIS OF VON WILLEBRAND DISEASE

With advances in understanding the molecular genetics of vWD, it is now possible to precisely diagnose and subclassify many variants of vWD on the basis of specific DNA mutations identified in the research laboratory. Unfortunately, DNA testing for vWD is not currently available in the clinical setting. As molecular testing is gradually introduced into the clinical laboratory, DNA diagnosis should be particularly straightforward for type 2B vWD, where a panel of four mutations detects over 80 percent of patients. Similar panels of mutations should be able to correctly identify the defect in the majority of type 2A and type 2N vWD. The analysis of type 3 and type 1 vWD will be more complex, since the currently known mutations account only for a small subset of these patients, except in selected populations.[131]

PLATELET-TYPE (PSEUDO-) VON WILLEBRAND DISEASE

Platelet-type (pseudo-) vWD is a platelet defect that phenotypically mimics vWD (see Chap. 119).[197] The plasma vWD lacks the largest multimers, RIPA is enhanced at low concentrations of ristocetin, and thrombocytopenia of variable degree is often present. Clinically, these patients have primarily mucocutaneous bleeding. Molecular analysis has identified mutations within the GPIbα chain as the molecular basis for pseudo-vWD. These mutations are located within the segment of GPIb thought to encode the vWF binding domain and appear to induce the conformational change complementary to that produced in the corresponding fragment of vWF by type 2B vWD mutations.[197]

The specialized RIPA test should be performed at low ristocetin concentrations to distinguish type 2B and platelet type vWD from type 2A vWD. Purified plasma vWF or cryoprecipitate causes platelet aggregation when added to platelet-rich plasma from patients with platelet-type vWD, distinguishing this disorder from type 2B vWD. In addition, type 2B vWD plasma transfers the enhanced RIPA to normal platelets, whereas plasma from patients with platelet-type vWD interacts normally with control platelets.

ACQUIRED VON WILLEBRAND DISEASE

Acquired vWD usually presents as a late-onset bleeding diathesis in a patient with no prior bleeding history and a negative family history of bleeding. Decreased levels of factor VIII, vWF:Ag, and ristocetin cofactor activity are common, and the bleeding time is usually prolonged. Acquired vWD is usually associated with another underlying disorder and has been reported to occur in patients with myeloproliferative disorders,[198] hypothyroidism,[199] benign or malignant B-cell disorders,[200] several solid tumors (particularly Wilm's tumor),[201] or certain

cardiac or vascular defects,[202] or in association with several drugs, including ciprofloxacin and valproic acid.[203,204]

A variety of B-cell disorders have been associated with the development of anti-vWF autoantibodies. In most cases the acquired vWD appears to be due to rapid clearance of vWF induced by the circulating inhibitor, although these antibodies may also interfere with vWF function. Hypothyroidism results in decreased vWF synthesis,[199] and, in some cases of malignancy, the acquired vWD is thought to be due to selective adsorption of vWF to the tumor cells. In acquired vWD associated with valvular heart disease or certain drugs, vWF may be lost by accelerated destruction or proteolysis.[203,204]

The vWF multimers in acquired vWD usually exhibit a type 2A pattern, with relative depletion of the large multimer forms. Distinguishing acquired vWD from genetic vWD can be difficult, since testing for the associated autoantibodies is generally not available in the clinical setting. The diagnosis often rests on the late onset of the disease, the absence of a family history, and the identification of an associated underlying disorder.

Management of acquired vWD is generally aimed at treating the underlying disorder. vWF levels and bleeding symptoms often improve with successful treatment of hypothyroidism or an associated malignancy. Refractory patients have been treated with corticosteroids, plasma exchange, intravenous gamma globulin, DDAVP, and vWF-containing factor VIII concentrates.[204]

THERAPY, COURSE, AND PROGNOSIS

The choice of treatment in any given patient depends upon the type and severity of vWD, the clinical setting, and the type of hemostatic challenge that must be met. A previous history of trauma or surgery and the success of previous treatment are important parameters to include in assessing the risk of bleeding. In general, the goals of therapy are to normalize the factor VIII activity and the bleeding time.

DESMOPRESSIN

Epinephrine, insulin, and vasopressin given to normal volunteers induce short-lived increases in factor VIII coagulant activity and vWF levels. Desmopressin (1-desamino-8-D-arginine vasopressin, DDAVP) is an analog of antidiuretic hormone and was originally produced for the treatment of diabetes insipidus. When DDAVP is administered to healthy subjects, it causes sustained increases of factor VIII and ristocetin cofactor activity for approximately 4 h.[205] DDAVP also releases tissue plasminogen activator and plasminogen activator inhibitor, presumably from endothelial cells. Patients with type 1 vWD treated with DDAVP release unusually high-molecular-weight vWF multimers into the circulation for 1 to 3 h after the infusion.[205,206] Therapy with DDAVP increases the factor VIII activity, vWF:Ag, and ristocetin cofactor activity to two to five times the basal level and, in many instances, corrects the bleeding time of type 1 vWD patients.

DDAVP has become a mainstay for the treatment of mild hemophilia and vWD.[207] It is regularly used in the setting of mild to moderate bleeding and for prophylaxis of patients undergoing surgical procedures. DDAVP is most commonly administered at a dose of 0.3 μg/kg, with an upper limit of 20 μg. Common side effects are mild cutaneous vasodilatation resulting in a feeling of heat, facial flushing, tingling, and headaches. The potential for dilutional hyponatremia, especially in elderly and very young patients, requires appropriate attention to fluid restriction, since it may result in seizures. There have been isolated reports of acute arterial thrombosis associated with administration of DDAVP, but the risk appears to be very low when judged against the total number of patients treated.

An intranasal form of DDAVP is also available and appears to be similar in efficacy to intravenous administration,[208,209] although the response may be more variable. Patients receiving DDAVP at closely spaced intervals of less than 24 to 48 h can develop tachyphylaxis. However, in one study, 22 type 1 vWD patients showed a departure of less than 20 percent from the mean factor VIII peak level calculated from two separate infusions. In addition, the consistency of response in one patient reliably predicted the future response of that patient and other affected family members.[210] For patients requiring repeated infusions of DDAVP, the factor VIII activity and vWF responses may not be of the same magnitude as after the first infusion. Although this decay in response has considerable individual variability, after one infusion of DDAVP per day for 4 days it was found that the responses on days 2 to 4 were reduced approximately 30 percent compared to day 1.[208-211]

Approximately 80 percent of type 1 vWD patients have excellent responses to DDAVP. In patients for whom DDAVP is potentially the treatment of choice, a test dose should be given (with measurements of before and after vWF and factor VIII levels) in advance of the first required course of treatment to ensure an adequate therapeutic response. For patients with type 1 vWD who are undergoing surgical procedures, DDAVP can be administered 1 h before surgery and approximately every 12 h thereafter. The response of factor VIII and ristocetin cofactor activity should be monitored when DDAVP is administered at frequent intervals. vWF-containing factor VIII concentrates and/or cryoprecipitate should be available for transfusion as backup.

Approximately 20 to 25 percent of patients with vWD do not respond adequately to DDAVP. This includes many type 2 vWD patients and nearly all patients with type 3 vWD. The response to DDAVP of patients with type 2A vWD is variable. Although most patients respond only transiently, some patients exhibit complete hemostatic correction after DDAVP infusion.[212,213] The differences in DDAVP efficacy among type 2A patients may correspond to the type of mutation, with better responses predicted in patients with group 2 mutations, although this hypothesis remains to be tested.

Many experts consider DDAVP to be contraindicated in the treatment of type 2B vWD, as the high-molecular-weight vWF released from storage sites has an increased affinity for binding to GPIb and might be expected to induce spontaneous platelet aggregation and worsening thrombocytopenia.[214] However, there are two reports of DDAVP used successfully in type 2B vWD patients, with an associated shortening or correction of the bleeding time and variable thrombocytopenia.[215,216]

VON WILLEBRAND FACTOR REPLACEMENT THERAPY

It is important to determine the response to DDAVP for each individual in order to avoid the unnecessary use of plasma products. For type 3 vWD patients and other patients unresponsive to DDAVP, the use of selected virus-inactivated, vWF-containing factor VIII concentrates is generally safe and effective.[217] Cryoprecipitate has been successfully used in the past, but since it is not currently treated to inactivate viruses, it is less desirable. Solvent-detergent-treated plasma is available, and cryoprecipitate prepared from such plasma may be an appropriate choice. It is important to note that most standard factor VIII concentrates are not effective for vWD, presumably because the vWF is either removed or undergoes degradation during processing. Only preparations that contain large quantities of vWF with well-preserved multimer structure are suitable for use in vWD patients. A recent study reported the analysis of 11 different factor VIII concentrates.[217] Humate P and VHP are currently the two most frequently used concentrates,

but only the former is available in the United States. Both of these concentrates have been shown to contain large vWF multimers resembling those found in normal plasma.[218,219]

Replacement therapy is largely empiric. In instances of serious bleeding or major surgical interventions, treatment may have to be repeated at least once a day. Although in general there is a correlation between normal hemostasis and correction of the bleeding time and factor VIII activity, this does not occur in all cases. In patients who have concomitant thrombocytopenia associated with or in addition to vWD, it may be necessary to transfuse platelets in addition to factor VIII concentrates. It is recommended that patients be treated for 7 to 10 days after major surgical procedures and for approximately 3 to 5 days after minor surgical procedures. Since postpartum hemorrhage can occur for up to a month or more after delivery, therapy may need to be prolonged in certain patients with severe disease. Sufficient therapy should be given to ensure normalization of factor VIII activity and shortening or correction of the bleeding time. If clinical bleeding continues, additional replacement therapy must be given and searches undertaken for other hemostatic defects. An occasional type 3 vWD patient will develop an alloantibody against the infused vWF, severely complicating replacement therapy.[220] The development of such a vWF inhibitor appears to be more common among type 3 vWD patients with large gene deletions.[26,126] A variety of approaches to the management of vWD inhibitors have been tried, including immunosuppression, similar to the treatment of factor VIII inhibitors in hemophilia A (see Chap. 123).

OTHER NONREPLACEMENT THERAPIES

Estrogens or oral contraceptives have been used empirically in treating menorrhagia. In addition to their effects on the ovaries and uterus, estrogens also tend to increase plasma vWF levels. Patients with vWD frequently normalize their levels of factor VIII, vWF:Ag, and ristocetin cofactor activity during pregnancy. The mechanism of action of estrogens may be related in part to the increased production of vWF through a direct effect on endothelial cells.[221] In pregnant patients with type 1 vWD, the factor VIII and ristocetin cofactor activities usually rise above 50 percent. These patients usually do not require any specific therapy at the time of parturition. In contrast, individuals who have 30 percent or less factor VIII or variant forms of vWD are more likely to require prophylactic therapy before delivery. Postpartum hemorrhage in all forms of vWD may occur as long as 1 month postpartum. Some patients are treated with plasma products prophylactically. Postpartum hemorrhage within the first few days after parturition may be related to the relatively rapid return to prepregnancy levels of factor VIII and vWF activities.

Fibrinolytic inhibitors such as ε-aminocaproic acid have been used effectively in some vWD patients. Fibrinolytic inhibitors have been suggested as an adjunct to DDAVP infusion, given the potential for enhanced fibrinolysis as a result of the release of tissue plasminogen activator along with vWF. However, fibrinolytic inhibitors are not generally used in this setting and are generally restricted to prophylactic treatment for dental procedures or empiric treatment of chronic menorrhagia or recurrent epistaxis.

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I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231, on January ___ 2005.

Judy Bridgwater

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:

Ton LOGTENBERG et al.

Serial No.: 09/284,107

Art Unit: 1639

Filing Date: (Int'l) October 7, 1997

Examiner: T.D. Wessendorf

For: METHODS AND MEANS FOR SELECTING PEPTIDES AND PROTEINS HAVING SPECIFIC AFFINITY FOR A TARGET

DECLARATION OF CORNELIS ADRIAAN (JOHN) DE KRUIF PURSUANT TO 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Cornelis Adriaan (John) de Kruif, declare as follows:

1. I am an inventor of the above-referenced patent application, and am familiar with the contents thereof.

2. I have a Ph.D. in molecular biology from Utrecht University received in 1996. I am currently a director of research and development at Crucell BV and have worked with proteins,

antibodies, and protein identification methods since 1988. I have expertise in the production, characterization of recombinant proteins, and methods to identify antigen-antibody interactions. A full curriculum vitae is attached as Exhibit A.

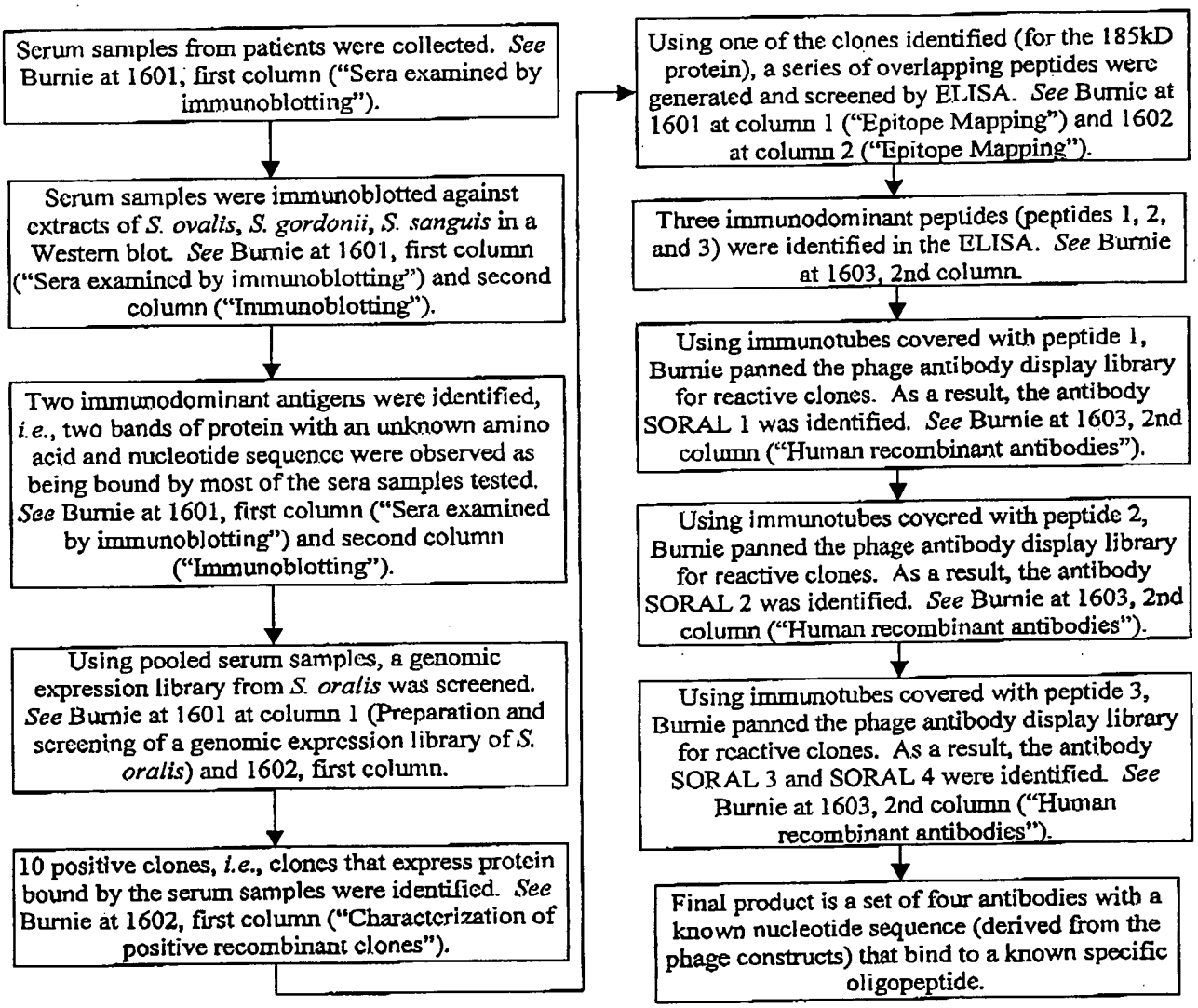
3. I have reviewed the assertions by the examiner regarding the cited combinations of de Kruif et al., *J. Mol. Biol.*, 248:97-105 (1995) in view of Geysen et al., *J. Immunol. Methods* 102:259 (1987) and Granoff, U.S. Patent No. 6,030,619; Burnie et al., *Infection Immunity* 64:1600-08 (1996); and U.S. Patent No. 6,265,150 (the '150 patent) and U.S. 2002/0132228 (the '228 application) in view of Middledorp et al (U.S. Patent No. 5,424,398).

4. A person of skill in the art would not be motivated to combine de Kruif with Geysen and Granoff or have a reasonable expectation that such a combination would work. de Kruif lacks any teaching regarding the use of oligopeptides libraries. The A2 domain is a large protein domain and is not equivalent to the short oligopeptides of the claimed methods. Geysen discloses a method that permits the identification of immunodominant epitopes of antigens using immune antiserum. Geysen lacks any teaching or suggestion regarding the identification of novel antibodies or their nucleotide sequences. Thus, to modify Geysen with the disclosure in de Kruif would destroy the intended purpose of the Geysen method. One of ordinary skill would not be motivated to make such modifications based on the teachings of the references.

Granoff is directed to a completely different concept than that of de Kruif, and thus no motivation is provided to make such changes. Basically, Granoff is directed to methods of identifying chemical compounds that mimic the native antigens of *Neisseria meningitidis* and can be used in a vaccine composition without eliciting autoreactive antibodies. Granoff seeks to identify these "molecular mimetics" for *N. meningitidis* by employing, *inter alia*, the identification of immunodominant epitopes using methods such as Geysen, *i.e.*, using immune antisera. Modifying Granoff to employ the plurality of antibody fragments of de Kruif would not help identify immunodominant epitopes that can elicit a protective immune response *in vivo* without eliciting autoreactive antibodies. Thus, one of ordinary skill would not be motivated to make such modifications based on the teachings of the references.

5. For very similar reasons, one of ordinary skill in the art would not combine the teachings of the '150 patent and the '228 application with Middledorp to result in the claimed methods. The '150 patent and the '228 application provide no teaching regarding the isolation of the nucleotide sequence of an antibody specific for a specific oligonucleotide sequence. The disclosure of both of these documents uses only whole cells to isolate antibodies. Middledorp simply discloses the identification of immunodominant viral proteins and their epitopes using immune antiserum. However, the methods in these documents are non-overlapping. The method taught in the '150 patent is not directed to identifying immunodominant epitopes while the method of Middledorp lacks any teaching or ability to identify novel antibodies or their nucleotide sequences. Because the modification of Middledorp results in the loss of its primary function, *i.e.*, to identify immunodominant epitopes, one of ordinary skill in the art would not combine these teachings.

6. Burnie is distinct from the claimed methods. The method disclosed in Burnie includes additional steps and lacks the ability to rapidly isolate the nucleotide sequences of multiple antibodies specific for specific, discreet peptides. The steps disclosed in Burnie are as follows:



The method disclosed by Burnie is complex, requires multiple steps of selection, multiple antibody sources for the selection process. The claimed methods, on the other hand, the rapid isolation of the antibody-encoding nucleotide sequence specific for a discreet peptide on a large scale without the use of immune antisera. Furthermore, the claimed method is not limited by

to the identification of immunodominant epitopes. Burnie fails to teach the method as claimed and lacks any motivation to climinate the multitude of additional steps included in Burnie to result in the claimed method. Thus, the person of ordinary skill in the art would have neither the motivation nor the reasonable expectation of success for such modifications given the disclosure of Burnie.

7. Taken together, nonc of the cited references in any combination motivate the skilled artisan to modify the teachings to result in thc claimed methods.

I hcrcby declare that all statements made herein of my own knowledgc are true and that all statements made on information and belief are believed to be true; and further that these statements were made with thc knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Executed at Leiden, Netherlands, on 04 Jan 05.

Cornelius Adriaan (John) de Kruif

PERSONAL DATA

Name	CA John de Kruif
Place of birth	Jutphaas, the Netherlands
Date of birth	7 Januari 1964
Nationality	Dutch

QUALIFICATIONS

1976 - 1982	Cals College, Nieuwegein, HAVO
1982	degree HAVO
1982 - 1987	Ghijssen Institute, Utrecht, HBO Biochemistry
1987	Bsc degree, Biochemistry
1989-1992	Utrecht University, Utrecht, Biology
1992	Msc degree, Biology
1992-1996	PhD program, Utrecht University, Faculty of Medicine, dep. Immunology
1996	PhD degree, subject 'Phage Display, selection and engineering of human antibody fragments'. (promotor Prof. HC. Clevers)

POSITIONS

1987-1992	research technician, Department of Veterinary Toxicology, Utrecht University
1992-1996	PhD student, Utrecht University, Faculty of Medicine, dep. Immunology
1996 - 1999	post-doc, Utrecht University, Faculty of Medicine, dep. Immunology
1999 - 2001	senior scientist, U-Bisys BV, later merged into Crucell BV
2001-	director R&D, Crucell BV

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